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# The Examination of Tubercle Bacilli in Sputum.

## II. A Concentration method of Tubercle Bacilli in Sputum. (A condensation method)

By

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(Received for publication, December 16, 1946)

### Foreword.

For the purpose of finding a very small number of tubercle bacilli in sputum, nothing is better than culture method in regard to sharpness. But to our regret, it will pass about a month before we have a distinct result by the use of this method, and for this reason we still recognize the value of concentration methods to a great extent.

We have had already many excellent methods of this kind, but as they had their faults mentioned afterwards, I have contrived a new method which is very convenient and effective.

### I. Some considerations of the concentration methods in the past.

Now we have many kinds of concentration method, but they may be roughly classified into four groups as follows: alkali-method, ferment method, heating method, and some other methods. As for the representatives of each class, we can mention Uhlenbhuth[1] and Tamura[2] as the representative of the alkali-method, Sullivan and Sears[3] as that of ferment method, Damen[4], and Pfeifer u. Robitschek[5] as that of the heating method, and Lange u. Nitsche[6], Hill[7], d'Arienzo[8], Kashiwagi[9], Sezaki[10], etc. are considered as the special methods, or mixed methods of more than two kinds of these special methods. Among these various methods, Tamura's[2], Pfeifer's[5] and Sezaki's[10] methods should be called the most excellent. The ferment method is out of question now. As Sezaki's[10] method is difficult and troublesome in using its instruments and in treatment, not only general medical practitioners but special medical men of

institutes with every equipment are unable to try this method.

The faults found not only in Tamura's[2] method but in every alkali method are the difficulty of fixing and dyeing of the smeared sputum and the rapid fading of sputum.

The heating method is used for the purpose of melting and making the sputum even, and this method is used almost in all cases together with the alkali method.

After all the object of all these methods is that we get a small amount of sediment by melting the sputum, and try to get a comparatively large amount of tubercle bacilli out of that sediment. But whatever method we may adopt in melting the sputum, the specific gravity of the solution becomes higher and higher, according to the degree of melting. And the specific gravity of the bacilli is 0950–1508 according to Pardo[11], and 1010–1080 according to Nebel[12] and it is easily understood that the specific gravity of the solution of sputum is not low, compared with that of the bacilli. Moreover, the solution has the viscosity of considerable degree, so it is quite natural that there are many numbers of tubercle bacilli which do not sink down by the use of a centrifugar that makes about 3,000 revolutions. This can also be clearly shown by the fact that the specific gravity of such solution as the emulsion of tubercle bacilli is small and, in case of some solution with very little viscosity, we can have considerable numbers of the tubercle bacilli that do not sink down under the strong centrifugalizing. Thus, in order to certificate the above fact of the sputum, I have made the following experiment.

## II. The comparison between the tubercle bacilli remaining in the upper part, and those in the sediment, of the solution of sputum.

First, I chose the sputum contained the bacilli which was discernible by microscopic examination, took up 2–3 cc of it and to this material added the equal quantity of the 4% solution of caustic soda liquid. After melting evenly, I mixed it up with 30 cc sterilized water, and centrifugalized it for a quarter of an hour, making 4,000 revolutions a minute. The sunken sediment obtained thus was not over 0.5 cc but was quite near 0.5 cc in almost all cases. So, I left 0.5 cc with a small amount of the upper part liquid in the centrifugal tube, and took out all the remaining upper part liquid. Then I mixed up each of these and from each of them, I cultivated 0.05 cc per piece on Oka-Katakura's culture medium. When too many bacilli were found in either of them, I thinned them with the addition of 2% caustic soda solution and then cultivated.

The result of this is shown in Table I. That is to say the case where the number of the tubercle bacilli contained in the upper part liquid is larger

TABLE I.

*The ratio between number of tubercle bacilli in upper liquid and that of the bacilli in centrifugal sediment.*

| Ratio | Number of examp. | % |
|---|---|---|
| more than same grade | 6 | 15.00 |
| the same grade | 6 | 15.00 |
| $\frac{1}{5}-\frac{1}{10}$ | 13 | 32.50 |
| $\frac{1}{10}-\frac{1}{100}$ | 10 | 25.00 |
| less than $\frac{1}{100}$ | 3 | 7.50 |
| 0 | 2 | 5.00 |
| Total | 40 | |

than that of the bacilli in the sunken sediment, amounts to 15% while the case where I can not certificate its existence in the upper part liquid is only 5%. As for the viscous sputum, the number of tubercle bacilli in the upper part liquid is always larger than the number in the sediment, or they are equal. And the case where the number of tubercle bacilli in the sunken sediment is less than 10 times the number of tubercle bacilli in the upper part liquid, is more than 60%. That is, in the method mentioned above, I cannot but conclude that the case where only 9 times the number of the tubercle bacilli is concentrated at best, is more than 60%. And as the upper part liquid is 32.5–33.5 and the sunken sediment 0.5 cc, one must know that the result of the culture on the upper part is naturally less satisfactory, when one takes 0.05 cc out of each part mentioned above. As 2–3 cc of the sputum is condensed to 0.5 cc practically, it is quite reasonable to believe that only 4–6 times the number of the tubercle bacilli can be concentrated, even if one can do it perfectly. As th tubercle bacilli remain in the upper part liquid of the sputum in this way, the percentage of concentration is, as a matter of fact, perhaps smaller. Tamura's method, the best of the alkali methods, is similar to the above-mentioned method after all, and the difference is only in the smallness of the quantity of alkali. Accordingly the sputum of 2–3 cc is condensed only to 0.5 cc. Inomata[13] says that he can concentrate only about twice as much in average in his practice of some other experiment on the application of Tamura's method. According to the above fact it is quite

imaginable that the large number of tubercle bacilli remain in the upper part liquid, if we take the alkali method of some other method in which we melt the sputum and divide it into two parts, the upper part liquid and the sediment.

### III. The method of practice.

If we wish to catch all the bacilli contained in the sputum from the beginning, we must not devide it into two parts, that is the upper part liquid and the sediment.

In order to condense the sputum easily and yet not to have any upper part liquid, it is better to give heat to the sputum. As for the heating method in the past, Ellerman u. Erlandsen[15] boiled the sputum with the caustic soda liquid and centrifugalize it. This method is similar to the alkali method. Biedert's[16] method is quite the same. The method by Pfeifer u. Robitschek[5] and Dahmen,[4] which is perhaps a true heating method, is after all a method of deviding the sputum into two parts, the upper part liquid and the sediment.

My own opinion may be most primitive, but as the greater part of sputum is water, I wish to condense the sputum by evaporating it.

Take some sputum in a evaporating dish—the deeper, the better—and heat it in a shallow water bath. Heating it for about 15–30 minutes—if the quantity of the sputum is large and thick, it is necessary to heat it about 1 hour–, one can condense the sputum to 1/5–1/15. One can condense it in this case better than in the case where one melts the sputum with alkali and centrifugalizes it. When one uses fine sands instead of water in the water bath, one can condense it in 5–20 minutes.

In both cases (especially in the latter) it is necessary to stir the sputum several times with a small, thin stick to keep it from scorching around the surface of the bath. The point is that one must treat the sputum so that it may have no dried part and also keep it always to be one lump.

Of course, one may make an ordinary smear preparation from this condensed sputum, but in this case one must take care not to smear too thick, andd let it dry of itself.

When one uses this method, the sputum does not lose its viscosity. Generally speaking difficulties to make preparations and microscope them will increase according to the decreasing degree of the quantity of the bacilli. But, adopting this method, it is easy to microscope because the sputum is to have the suitable thickness.

### IV. The Results.

The results obtained by the above-mentioned method are shown in Table II and III.

In Table III use the sputum which contains the bacilli of the middle class thickness or of small amount. In the experiment of the above-mentioned sputum, there are few cases that the number of the tuberculous bacilli discovered after the condensation shows, like the experiment shown in the section II, the great difference compared with the number of the bacilli, discovered through non-condensation method. But, in the sputum which contains very small number of the tubercle bacilli or in the negative sputum, there appears a great difference as shown in Table III.

Now I am going to make an explanation of Table III. Among the 16 examples which are negative, 10 became positive. The examples 11, 13, 15 and 18 are the results of the experiments of 2 mornings' sputum; the examples of 5th and 13th and of 8th and 11th are the results of the experiments of the sputum from the same person. Though examples are not many, yet it seems that I can have good results. And in the examples of 6th, 7th, 9th, 15th, 17th, and 18th, I could testify bacilli usually only by culture.

In case of the sputum, the condensation rate of which is about 1/5, shown in Table II and III, I used a shale instead of an evaporating-dish.

TABLE II.

*Sputum that contains few of bacilli.*

| I | II | III | IV | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|
| Examp. | Quantity of sputum | Sputum-volume after condensation | II: III | Visual fields | Number of bacilli in non condensed sputum | Number of bacilli in condensed sputum | VI: VII |
| 1 | 1.5 cc | 0.3 cc | 5:1 | 100 | 114 | 393 | 1:3 |
| 2 | 1.5 | 0.3 | 5:1 | 50 | 175 | 720 | 1:4 |
| 3 | 1.0 | 0.2 | 5:1 | 50 | 34 | 131 | 1:4 |
| 4 | 1.0 | 0.3 | 3:1 | 50 | 15 | 64 | 1:4 |
| 5 | 2.0 | 0.5 | 4:1 | 50 | 21 | 106 | 1:5 |
| 6 | 2.5 | 0.5 | 5:1 | 50 | 3 | 25 | 1:8 |
| 7 | 5.0 | 0.2 | 25:1 | 100 | 6 | 58 | 1:10 |
| 8 | 3.0 | 0.5 | 6:1 | 100 | 8 | 92 | 1:11 |
| 9 | 4.0 | 0.5 | 8:1 | 50 | 54 | 1.041 | 1:20 |
| 10 | 4.0 | 0.5 | 8:1 | 100 | 8 | 174 | 1:22 |
| 11 | 2.0 | 0.5 | 4:1 | 50 | 17 | 302 | 1:24 |

TABLE III.

*Sputum that contains very few or no bacilli.*

| I | II | III | IV | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|
| Examp. | Quantity of sputum | Sputum volume after condensation | II: III | Visual fields | Number of bacilli in noncondensed sputum | Number of bacilli in condensed sputum | VI: VII |
| 1 | 3.0 cc | 0.5 cc | 6:1 | 100 | 1 | 1 | 1:1 |
| 2 | 0.5 | 0.2 | 3:1 | 100 | 1 | 4 | 1:4 |
| 3 | 6.0 | 0.6 | 10:1 | 100 | 1 | 12 | 1:12 |
| 4 | 0.5 | 0.1 | 5:1 | 50 | 2 | 74 | 1:37 |
| 5 | 2.0 | 0.5 | 4:1 | 100 | 0 | 0 | 0:0 |
| 6 | 2.0 | 0.2 | 10:1 | 100 | 0 | 0 | 0:0 |
| 7 | 2.0 | 0.2 | 10:1 | 100 | 0 | 0 | 0:0 |
| 8 | 1.0 | 0.2 | 5:1 | 100 | 0 | 0 | 0:0 |
| 9 | 1.5 | 0.1 | 15:1 | 100 | 0 | 0 | 0:0 |
| 10 | 10.0 | 0.6 | 17:1 | 100 | 0 | 0 | 0:0 |
| 11 | 1.5 | 0.1 | 15:1 | 100 | 0 | 3 | 0:3 |
| 12 | 2.0 | 0.5 | 4:1 | 100 | 0 | 4 | 0:4 |
| 13 | 6.0 | 0.5 | 12:1 | 100 | 0 | 6 | 0:6 |
| 14 | 5.0 | 0.5 | 10:1 | 100 | 0 | 8 | 0:8 |
| 15 | 5.0 | 0.4 | 12:1 | 100 | 0 | 8 | 0:8 |
| 16 | 1.0 | 0.2 | 5:1 | 100 | 0 | 14 | 0:14 |
| 17 | 2.0 | 0.5 | 4:1 | 100 | 0 | 15 | 0:15 |
| 18 | 2.0 | 0.2 | 10:1 | 100 | 0 | 27 | 0:25 |
| 19 | 1.0 | 0.1 | 10:1 | 100 | 0 | 126 | 0:126 |
| 20 | 5.0 | 0.5 | 10:1 | 150 | 0 | 192 | 0:192 |

But it took a considerably long time and yet condensation was not satisfactory.

## SUMMARY.

Thus I have contrived a method of concentration by which I can produce a good result without any high speed centrifuger or other special

furnishment. That is to say, it is a method that one takes sputum in evaporating-dish and heat and condense it. The method is quite convenient and practicable to any general medical men.

Thus, by this method, they can condense the sputum to 1/5–1/15 size of the original. Among the 16 experiments of the negative sputum I could find the bacilli in 10 examples; among 6 examples in which the bacilli could be seen only by culture, the existence of the bacilli could be testified in 3 examples.

In this method, the fixation and dyeing of the smeared sputum is quite the same as the preparation of ordinary speciment.

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# Close Relation between Prothrombin Time and Thiamin; Contribution to the Study of Normal Prothrombin Time. Preliminary Report.

By

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The close relation between vitamin K deficiency and hypoprothrombinemia is well known. But as to the intimate relation between prothrombin time and thiamin, there has been no report within our knowledge. Here we shall report preliminarily on this relation.

It was rather accidental that we could notice the alleged intimate relation. We had been investigating prothrombin time of some diseased children, but we had used healthy lactating mothers as control, *irrespective of their Arakawa's reaction*[1]: (Negative reaction is a sign of thiamin deficiency) —because we had thought that even Arakawa-negative mothers, apparently healthy, could serve as controls as well. In the course of investigation, however, suspicion was aroused that prothrombin time might possibly be prolonged in them. Hence a systematic investigation into the relation between prothrombin time and Arakawa's reaction, or into the relation between prothrombin time and thiamin deficiency.

We studied it in 270 cases of lactants[2] and found that Arakawa-negative mothers—lactants with thiamin deficiency—had a more or less prolonged prothrombin time, further that thiamin supply to such cases brought it down to the normal. On the other hand we examined 200 cases of breast-fed infants nursed with milk of different Arakawa's reaction and found that those nursed with Arakawa-negative milk showed a more or less prolonged prothrombin time. Further we found that thiamin administration brought it down to the normal. It was not rare that prothrombin time, apparently normal, was shortened by 4 or 5 seconds by thiamin injection.

*Thus, women in labour ought not be suffering from vitamin K deficiency, but also from thiamin deficiency either.*

## Literature.

1) Cf. many Reports on the Peroxidase Reaction published in this Journal since 1930. 2) K. Sakai, Tohoku J. Exp. Med., (220th and 221st Reports of the Peroxidase Reaction) to be published in a fourthcoming issue. 3) K. Hayashi, Ibid. (243rd and 245th Reports on the Peroxidase Reaction) to be published in a forthcoming issue.

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# Über die Tuberkuloseinfektion und die Allergie im Säuglings- und Kindesalter.

Von

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Bekanntlich hat V. Behring[1] vor ungefähr 50 Yahren den Ausspruch getan, dass die Entwickelung der Lungenphthise im frühesten Kindesalter beginnt und die Lungenschwindsucht des Erwachsenen die letzte Strophe des Liedes ist, welches an der Wiege des Betroffenen gesungen wurde. V. Behring's ursprüngliche Ansicht war die, dass das Kind durch das Trinken von Kuhmilch enteral infiziert und die hier einmal festgesetzten Tuberkelbazillen später im Pubertätsalter oder noch später ihre zerstörende Wirkung entfalten. Diese Ansicht ist heute nicht in ursprünglicher Form haltbar, weil der Erreger der Lungentuberkulose Typus humanus ist, während der in der Kuhmilch enthaltene Bazillus Typus bovinus ist. Die heutzutage vorherrschende Meinung, im Grunde nicht anderes als eine etwas veränderte Form der V. Behringschen Ansicht, ist dass jeder im Kindesalter einmal von Tuberkelbazillen infiziert wird und danach im Erwachsenenalter endogen oder exogen eine neue Infizierung von Tuberkelbazillen eintritt, infolgederen sich die Lungenschwindsucht entwickelt. Die meisten Autoren stellen sich die unmittelbare Entwickelung der Lungenphthise von der Primärinfektion aus nur im Kindesalter möglich und im Erwachsenenalter als Ausnahme vor. Dagegen wird in der nördlichen Gegend Europas, Schweden und Norwegen, so wie auch in Japan, die unmittelbare Entwicklung der Lungenschwindsucht als nicht selten angesehen, sogar behaupten die japanischen Autoren, die sich mit diesem Problem beschäftigt haben, dass eine derartige Entwickelung der Lungenphthise die Regel ist. Durch die Infektion von Tuberkelbazillen wird der Organismus allergisch. Die herrschende Ansicht ist die, dass die Allergie, die sich hauptsächlich als Tuberkulinsensibilität manifestiert, 5–6 Wochen nach der Infektion beginnt und lebenslang positiv bleibt und nur im seltensten Fall negativ wird, wenn die Krankheit vollständig geheilt und

die Tuberkelbazillen im Organismus gänzlich vernichtet werden. Dies sind Fälle der positiven Anergie; ausserdem gibt es Fälle negativer Anergie bei fortgeschrittener oder heruntergekommener Phthise oder bei interkurrenten Krankheiten, wie bei Masern und Keuchhusten. Es sind aber neuerdings mehrere neue Tatsachen berichtet worden, welche mit dieser traditionell gewordenen Ansicht nicht im Einklang stehen. Andererseits ist man inzwischen mit der Nachweismethode der Tuberkelbazillen vorwärts gekommen. Früher wurde der Tierversuch hauptsächlich zum Nachweis der Tuberkelbazillen angewendet. Seitdem die Kultivierung von Tuberkelbazillen mittels des Eiernährbodens in die Klinik eingeführt worden ist, hat diese wegen ihrer Einfachkeit und Sicherheit rasch grosse Ausbreitung gefunden. Und sie ist inbezug auf die Sicherheit keinesweges dem Tiereversuch unterlegen. Ligner[2] konnte bei einem 4 Monate alten Kinde, das von tuberkulösen Eltern abstammte in der Magenspülflüssigkeit mittels des Meerschweinchenversuches Tuberkelbazillen schon in der Periode nachweisen, wo die intrakutane Tuberkulinreaktion noch negativ war. Nachträglich hat sich die Tuberkulinreaktion in positive Reaktion umgewandelt. Die Periode, wo die Tuberkuloseinfektion sicher stattfand und noch nicht allergisch wurde, nannte er praeallergische Periode der Tuberkuloseinfektion. Übrigens konnten Armand-Delille und Vibert,[3] so wie auch Poulssen[4] die mit der von Meunier[5] vorgeschlagenen Methode die Ventrikelspülflüssigkeit bakteriologisch untersuchten, in zahlreichen Fällen Tuberkelbazillen von früherer Primärinfektion der Kinder nachweisen. Wallgren[6] untersuchte 40 Kinder mit Erythema nodosum, das bekanntlich zum spezifischen Symptome der Primärinfektion gehört. Dabei fand er 37 Fälle tuberkulinpositiv, von denen er bei 17 Tuberkelbazillen nachweisen. Leon Bernard und Long[7] hatten Gelegenheit zu anatomischer Untersuchung eines beim Tod 2 Monate alten von seiner Mutter tuberkulös infizierten Kindes, das vor Eintritt der Allergie an Cholera infantium gestorben war. Es bestanden keine makroskopischen tuberkulösen Veränderungen, weder in den Lungen noch in den Hilusdrüsen. Verimpfung von Material aus Lymphdrüsen, Milz und Leber auf Meerchweinchen ergab indes positives Resultat für Tuberkulose. Auch Opitz[8] konnte bei Kindern mittels des Tierversuches Tuberkelbazillen im Magenspülwasser nachweisen. Obwohl der Autor bei der Untersuchung nicht speziell die Primärinfektion im Auge hatte, so finden sich unter seinem Material Fälle von Primärinfektion, die nach den mitgeteilten Röntgenbildern ziemlich fortgeschritten sind. Dass der Tuberkelbazillus schon im Stadium der Primärinfektion im zirkulierenden Blut nachweisbar ist, haben Kumagai, Iibuchi und Ogawa[9] mittels der Kulturmethode festgestellt. Sato[10] konnte dabei Tuberkelbazillurie nachweisen. Katakura und Oka[11]

konnten bei 5 Fällen unter 25 erwachsenen Patienten mit Primärinfektion kulturell Tuberkelbazillen nachweisen. Auch konnte Naranjo[12] bei 34 unter 131 Fällen von Erythema nodosum im Kindesalter mittels des Tierversuches Tuberkelbazillen im Blut nachweisen. Die Bazillaemia soll auch vor der Tuberkulinreaktion auftreten und kann sich über 4 Wochen hinziehen. Ein ähnlicher Fall wird von Kumagai, Iibuchi und Ogawa[9] mitgeteilt: bei einem 26 jährigen tuberkulinnegativen Fall wurden Tuberkelbazillen aus Blut kultiviert. Nachträglich fanden sie bei Durchleuchtung und photographischer Aufnahme in der Schrägenstellung des Patienten eine vergrösserte Lymphdrüse im Mediastinum. Oka[13] verfolgte mittels systematischer Untersuchung bei 293 Schülern einer Normalschule während zweier Yahre die Infektion mit Tuberkelbazillen. Unter 19 Schülern, deren Tuberkulinreaktion im Anfang negativ war und sich inzwischen in positiv umwandelte, konnte er bei 7 Fällen (36.8%) Tuberkelbazillen aus dem Auswurf kultivieren. Ausserdem konnte er bei 14 Fällen unter den 32 erwachsenen Patienten der Primärinfektion, die poliklinisch oder klinisch beobachtet wurden, Tuberkelbazillen aus dem Sputum kultivieren, also bei 43.7%. Nach den mitgeteilten Untersuchungsergebnissen ist sicher festgestellt, dass Tuberkelbazillen im frühesten Stadium der tuberkulösen Infektion sowohl bei Kindern als auch bei Erwachsenen im zirkulierenden Blut vorhanden sind und im Auswurf ausgeschieden werden. Was die Allergie und zwar die Tuberkulinsensibilität anbetrifft, so glaubte man, dass sie lebenslang manifest bleibt, wenn sie einmal erworben ist. Neuerdings werden aber Tatsachen mitgeteilt, die gegen diese Ansicht sprechen. Wer mit einem schwachvirulenten Tuberkelbazillus Tierversuch angestellt hat, der hat wohl erfahren, dass die einmal positiv gewordene Tuberkulinreaktion bei Meerschweinchen, Kaninchen oder Affen sehr leicht schwindet. Amenille und Caneti meinen, dass entgegen der älteren Ansicht das Negativ-werden oder positiven Tuberkulinreaktion, d. h. die vollkommene Heilung der latenten Tuberkuloseformen auch beim Menschen in der Mehrzahl der Fälle (80%) eintritt. Die Tatsache, dass trotzdem bei fast allen Erwachsenen positive Reaktion gefunden wird, erklären sie aus dem, Wiederaufflammen der Sensibilität infolge fortgesezter Reinfektion. Leon Bernard und Debre[14] beobachteten, dass manche Fälle positiver Tuberkulinreaktion negative werden. Nach Bezancon[15] kann es sich vielleicht um eine ganz leichte oder unvollkommene Infektion handeln, sodass sie in kurzer Zeit vollkommen geheilt wird. Es gibt auch Mitteilungen, vor allem aus den Vereinigten Staaten von Amerika, dass viele mit deutlichen Kalkschatten im Röntgenbild tuberkulinnegativ sind, ein Zeichen dafür, dass viele Fälle auch manifester Tuberkuloseinfektion vollständig geheitlt werden. (Crimm, Darwin und Short,[16] Dearing,[17] Goss, Harrison,

Stewart und Williams[18] Merchandise,[19] so wie Paisseau, Valtis und Debre, Valtis und Deise[20] berichten, dass es Kinder gibt, die Sputum Tuberkelbazillen beherbergen und doch tuberkulinnegativ bleiben. Dies erklären sie dadurch, dass die Allergie durch fortgesetzte Aussetzung der Bazillen negativ bleiben, oder es ist eine prolongierte Anteallergie. Es ist auch möglich, dass die Empfindlichkeit der menschlichen Haut an und für sich veränderlich ist. Ist dies der Fall, so ist die Tuberkulinreaktion an sich nicht so genau zu nehmen.

Um die oben auseinandergestzte Frage zu beleuchten haben wir eine systematische Untersuchung an Kindern eine Waisenhauses vorgenommen. In diesem sind in der Regel etwa 75 Kinder untergebracht. Die Zahl der Pfleglinge schwankt fortwährend, weil jederzeit neue hinzukommen, weggebracht werden oder sterben. Es gibt recht viele Kinder, die von tuberkulösen Familien abstammen, insbesondere von tuberkulösen Müttern, die gestorben sind. Deswegen konnten wir nur bei 37 Kindern während eines ganzen Jahres vollkommene Untersuchung anstellen. Ausser der Messung der Körperlänge, des Körpergewichts, der Körpertemperatur, des Pulses und der gewöhnlichen Untersuchung, wie Percussion und Auskultation, wurden Blutsenkung nach Westergren vorgenommen, Röntgenaufnahme gemacht und intrakutane Tuberkulinreaktion festgestellt. Diese letztere wurde mit 1: 1000 Alt-tuberkulin geprüft, wenn negativ mit 1: 100 Verdünnung, und nach 24 resp. 48 Studen abgelesen. Zu gleicher Zeit wurde Kultivierung von Tuberkelbazillen aus Sputum, Rachenabstrich und aus dem Magensaft resp. Magenspülwasser der nüchteren Zeit vorgenommen. Diese Untersuchungen wurden im Abstand von 3 Monaten wiederholt und während eines ganzen Jahres fortgesetzt. Wenn man unter den genau untersuchten 37 Fällen diejenigen herausnimmt, deren intrakutane Tuberkulinreaktion sowohl mit 1: 1000 wie auch 1:100 Verdünnung negativ sind, so bleiben 15 Fälle. Unter diesen haben wir in 10 Fällen bei 3 resp. 6 maliger Aufnahme kein abnormes Röntgenbild der Brust bekommen. Auch 3 bis 6 mal wiederholte Kulturversuche der Tuberkelbazillen ergaben bei diesen 10 Fällen immer negatives Resultat. Diese 10 Fällen kann man wohl mit Recht zu den noch nicht mit Tuberkelbazillen infizierten rechnen. Bei 4 von den 15 Fällen traten im Röntgenbild während der Beobachtung abnorme Schatten auf, die dem infiltrativen Lungenprozess entsprechen. Die 4 Fälle reagierten auf die intrakutane Tuberkulininjektion gar nicht, auch mittels 1: 100 verdünnten Tuberkulins. Sie sind gestorben und wurden seziert. Trotz der genauen makroskopischen und mikroskopischen Untersuchung wurde keine tuberkulöse Veränderung, sondern croupöse resp. katarrhalische Pneumonie gefunden. Der letzte Fall von diesen 15 tuberkulinnegativen Fällen ist

sehr interessant. Bei diesem wurden die Tuberkelbazillenkultur 5 mal angestellt. Der am 1. Nov. 1939 vorgenommene Kulturversuch aus Rachenabstrich ergab ein positives Resultat. Die an diesem Tage aufgenommene Röntgenphotographie zeigte ein hilusnahes Infiltrat. Das 2 Monate nachher aufgenommene Röntgenbild war fast dasselbe. Die intrakutane Tuberkulinreaktion war bei diesem Fall vom November 1939 bis November 1940 8 mal untersucht und immer negativ gefunden worden. Für eine einfach Praeallergie ist die Zeit zu lang. Sonst war das Kind gut genährt und munter. Als eine negativ Anergie war er gar nicht zu denken. Eher kann man sich denken, dass die Infektion mit Tuberkelbazillen unter Umständen, bei denen es sich wohl um eine inkomplete Injektion handeln würde, ohne Allergie verlaufen kann. 5 Fälle unter 37 Untersuchten reagierten vom ersten Versuch an auf die intrakutane Injektion von 1: 1000 verdünntem Alt-Tuberkulin mittelstark bis stark und alle Male positive. Alle diese Fälle zeigten auch abnorme Veränderung des Röntgenbildes der Brust. Bei sämtlichen 5 Fällen sind Tuberkelbazillen gezüchtet worden. Bei einem Fall, dessen Röntgenbild haematogene Streuung vorwies, wurden sie aus Liquor cerebrospinalis herausgezüchtet. Dieser Fall und ein weiterer mit einem Röntgenbild haematogener Streuung sind gestorben, und die tuberkulöse Infektion wurde durch die Sektion bestätigt. So ist der Tuberkelbazillus bei allen deutlich allergischen Kindern mit abnormen Röntgenbildern nachgewiesen. Umgekehrt könnte man sagen, dass im Säuglings-und Kindesalter der Nachweis einer stark positiven Tuberkulinreaktion mit offener Lungentuberkulose identisch ist. Es sind 17 Fälle, deren intrakutane Tuberkulinreaktion mit 1: 1000 A. T. bei der ersten Untersuchung negativ war, aber mit der 1: 100 Verdünnung A. T. bei der ersten Untersuchung oder später einmal positiv gewesen ist. Bei 4 Fällen wurde diese Reaktion auch mit der Verdünnung 1: 1000 vorübergehend positiv. Es war ziemlich charakteristisch, dass die positive Umwandlung der Tuberkulinreaktion niemals stark war, meist $6\times6-15\times15$ mm. Ferner war es sehr auffallend, dass diese Tuberkulinempfindlichkeit nicht stetig, sondern sehr veränderlich war: bald trat sie auf, bald verschwand sie. Man konnte nicht gewiss nachweisen, ob die positive Umwandlung der Tuberkulinreaktion endgültig war oder nicht. Es ist möglich, dass die Untersuchung gerade in der positiven Phase abgebrochen wurde. Bei 10 von diesen 17 Fällen war das Röntgenbild des Thorax abnorm verändert; 7 Fälle hatten Hilusdrüsenschwellung, bei einem Fall wurde ein deutlicher bipolarer Schatten bemerkt, der für die Primärinfektion charakteristisch ist. Bei 7 Fällen unter den 10 mit abnormalen Röntgenbildern wurden die Tuberkelbazillen bald aus Rachenabstrich allein, bald aus Rachenabstrich und Magensaft zugleich gezüchtet. Bei den 7 Fällen mit abnormen

Röntgenbefund, positiver Tuberkelbazillenkultur und positiver, wenn auch nur zeitbegrenzter, Unwandlung der Tuberkulinreaktion hat tuberkulöse Infektion höchstwahrscheinlich stattgefunden. Auch bei den 3 Fällen, die Hilusdrüsenschwellung oder Infiltrat im Röntgenbild zeigten, bei denen zwar keine Tuberkelbazillen nachgewiesen konnten, aber vorübergehende positiv Umwandlung der Tuberkulinreaktion bemerkt wurde, hat wahrscheinlich Tuberkuloseinfektion stattgefunden. Die Tuberkuloseinfektion, wie man sie hier sieht, ist vielleicht eine sehr leichte oder inkomplete Infektion, sodass der eingedrungene Tuberkelbazillus in kurzer Zeit vertilgt wird. Leichte Infektion findet man bei allen Infektionskrankheiten, und sie sind in der Regel zahlreicher als die Fälle mit typischer Infektion, eine Tatsache, die bei Abdominaltyphus, Diphtherie u.s.w. sehr bakannt ist. Also in der Umgebung des typisch Infizierten gibt es zahlreiche leichte, atypische und inkomplete Fälle. Es kann auch einfach gesunde Tuberkelbazillenträger geben. Nach der heutzutage herrschenden Ansicht über die tuberkulöse Infektion dringt der Tuberkelbazillus in den meisten Fällen auf dem Luftwege in der Bronchiolus oder in die Alveolen der Lunge und setzt sich dort fest. Daselbst entwickelt sich ein bronchopneumonischer Herd, wo die entsprechende Lymphdrüse affiziert wird. Nach der Heilung hinterlässt dieser einen verkalkten oder verknöcherten mit hyaliner Bindegewebekapsel umgebenen Herd, der dem harten Primärkomplex entspricht. Die Feststellung des Primärkomplexes durch Kuss[21] E. Albrecht,[22] H. Albrecht,[23] Gohn,[24] Ranke[25] u.s.w. ist eine fundamentale Entdeckung. Was ausser diesem typischen Primärkomplex bei einer leichten Infektion hinterlassen wird, weiss man nicht genau. Es kann Narbe, Induration oder atelektatische Narbe sein. O. Naegeli[26] hat vor 30 Yahren an dem Beobachtungsmaterial des Züricher pathologischen Instituts festgestellt, dass fast jeder Erwachsene, der zur Sektion kam, Spuren einer früher durchgemachten tuberkulösen Infektion auswies. Aus Zahlreichen Beobachtungen weiss man jetzt, dass Durchseuchung der Bevölkerung örtlich verschieden ist und sie sich im letzten Jahrzehnt mehr nach dem Erwachsenenalter hin verschoben hat. Es ist selbstverständlich, dass durch Verminderung der offenen Tuberkulose, die sowohl durch die Fortschritte der Behandlungsmethoden, wie die Kollapstherapie, als auch durch Besserung der Lebensverhältnisse herbeigeführt wurde, die Gelegenheit zur Infektion vermindert worden ist. Trotzdem taucht die Frage immer wieder auf, ob alle im erwachsen Alter Tuberkulinnegativen wirklich nicht infiziert sind oder, obwohl einmal infiziert, die Allergie verloren haben. Für die letztere Ansicht, dass es unter den tuberkulinnegativen Erwachsenen manche gibt, die einmal tuberkulös infiziert, aber vollständig geheilt wurden, spricht die Tatsache, dass die einmal erworbene Allergie,

wie sie in der Einleitung angeführt ist, oft verloren geht. Dafür spricht weiter, dass viele Fälle, die röntgenologisch deutliche Kalkherde in der Lunge zeigen, tuberkulinnegativ sind. Z. B. fanden Crimm und Short[16] 191 solche Fälle unter 1384. In Verlauf von Jahren wurden 4 Fälle wieder allergisch oder reinfiziert. In 14 Fällen wurde ein Verlust der Allergie festgestellt. Ähnliche Mitteilungen gibt es viele. Auch fehlt es nicht an Angaben, dass die Lungenkalkherde nicht nur bei der Tuberkulose, sondern auch bei Embolie, Thrombus, Siderosis, Blastomykosis und a.m. wie schon erwähnt, vorkommen. Aber solche Fälle gehören wahrscheinlich zur Seltenheit. Amenille und Caneti[27] gehen so weit zu behaupten, dass das Verschwinden der Tuberkulinreaktion in der Mehrzahl der Fälle (etwa 80%) eintritt. Die obige Ansicht, dass die einmal erworbene Primärinfektion vollständig geheit und das typische Röntgenbild verschwindet, aber im Röntgenbild wieder bipolarer Schatten und positive Tuberkulinreaktion mit typischen Symptomen der Primärinfektion auftraten wird durch die genau beobachteten Fälle von Kumagai[28] und Ickert[29] bestätigt. Nach den hier mitgeteilten Untersuchungsergebnissen ist die Möglichkeit nicht ausgeschlossen, dass leichtgradige Infektion mit Tuberkelbazillen ein tägliches Geschehen sind, d.h. dass die Stadtbewohner sich öfter in ihrem Leben infizieren und deshalb die Tuberkulinallergie lebenslang beibehalten. Die Leute, die selten die Gelegenheit zur Tuberkuloseinfektion ausgesetzt sind, wie die Landbewohner, werden von der Tuberkuloseinfektion leicht geheilt. Wenn aber die Tuberkuloseinfektion einen gewissen Grad überschritten hat, so ist sie im gewissen Sinne unheilbar.

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# Wirkungen der verschiedenen Vitamine auf den Glykogenbestand der Leber unter besonders warmer Bedingung.

Von

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## I. Einleitung.

Es ist schon von manchen Forschern bekannt gemacht worden, dass das Vitamin C und die ihm verwandten Stoffe nicht nur antiskorbutisch, sondern auch auf den Kohlehydratstoffwechsel fördernd zu wirken imstande sind; für diesbezügliche nähere Literatur verweise ich auf die schon publizierten Arbeiten aus unserem Institut (Terada,[1] Simizu,[2] Watanabe,[3] Takezawa[4] und Miyakosi[5].

Nach den Arbeiten von Terada[1] und Takezawa[4] wirken Vitamin C und die ihm verwandten Stoffe (z. B. der rohe Pressaft der verschiedenen Obstarten und verschiedene chemische Stoffe wie l-Matteucinol, Homoeryodictyol, 7-Oxyflavonon usw.) auf den Glykogenbestand der Kaninchenleber vermehrend und zwar fixierend. In diesem Sinne behält der Obstsaft diese Wirkung auch dann bei, wenn das C-Prinzip von der Hitze zerstört wird. Deshalb sind wir ungezwungen zu dem Schluss gekommen, dass man im Obstsaft noch ein anderes wirksames Prinzip ausser C-Vitamin annehmen muss.

Ich unternehme hier einen Versuch, in dem ich bestimmen will, wie das Vitamin P bzw. K im Vergleich mit C auf den Glykogenbestand der Kaninchenleber im normalen bzw. besonders warmen Zustand wirkt.

## II. Versuchstechnik.

Um Versuchstiere zu sparen und ausserdem auch zum Zwecke, mit einem und demselben Individuum fortwährend die Veränderung des Glykogenbestandes verfolgen zu können, wendete ich eine neue Methode an, die Prof. Kurokawa und seine Mitarbeiter[6] in hiesiger Klinik bear-

beitet hatten; nämlich ein gesundes Kaninchen, dem vorher die beiderseitigen Intercostalnerven (VIII, IX, X, XI) durchschnitten worden waren, damit das Tier bei der Bauchoperation keine Schmerzen mehr empfinden sollte; wurde laparotomiert und unter der Kontrolle des unbewaffneten Auges von der Leber ein minimales Stückchen mit einer kleinen Klemmschere fast ohne nennenswerte Blutung herausgeschnitten und von diesem Leberstückchen wurde der Gehalt an Glykogen analysiert. Die quantitative Glykogenbestimmung geschah wie folgt: das herausgeschnittene Leberstückchen (ca. 10 mg) wurde in ein Reagenzglas getan, das ca. 1 ccm 30 %iger Kalilaugelösung enthält und im siedenden Wasserbade vorher erwärmt ist und weiter unter dem Rückflusskühler 30 Min. lang bearbeitet. Nach dem Erkalten wurde es mit 10 %iger Salzsäurelösung genau neutralisiert, dann in schwach saurer Lösung (ca. 2%) 30 Min. lang hydrolisiert, wieder neutralisiert, mit Cadmium-sulfatlösung und normaler Kalilaugelösung enteiweisst. Mit dem Filtrat wurde die reduzierende Kraft analysiert und von dieser der Glykogenwert berechnet. Zu diesem Versuche wurden als Vitamin C Redoxon " Roche" 100 mg pro kg Körpergewicht, als P-Vitamin 5 mg pro kg Hesperin „Takeda" und als K-Vitamin 10 mg Katiev „Takeda" gebraucht.

### III. Versuchsergebnisse.

Die gesamten Versuche gestalteten sich wie folgt.

A. Bei normalen Kaninchen.
   1. Kontrollversuch ohne jegliche Behandlung.
   2. Versuch mit alleiniger Anwendung von Glukoselösung.
   3. Versuch mit alleiniger Anwendung von Vitaminarten.
   4. Versuch mit einer Mischung von Glukose mit Vitamin C.
   5. Versuch mit einer Mischung von Glukose mit Vitamin P.
   6. Versuch mit einer Mischung von Glukose mit Vitamin K.
   7. Versuch mit der kombinierten Anwendung von Glukose mit Vitamin C, P und K zusammen.

B. Unter der Einwirkung hoher Temperatur (42°C).
   1. Kontrollversuch.
   2. Versuch mit Glukose allein.
   3. Einwirkung der Glukose und des Vitamins C.
   4. Einwirkung der Glukose und des Vitamins P.
   5. Einwirkung der Glukose und des Vitamins K.
   6. Einwirkung der kombinierten Anwendung der Glukose mit Vitamin C, P und K zusammen.

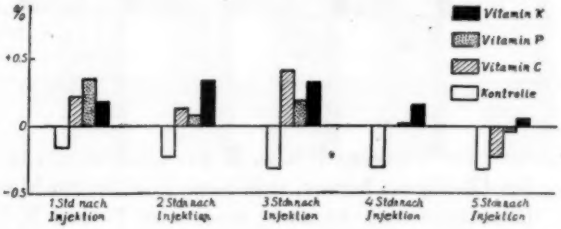
Es ist ziemlich umständlich, diese 13 hintereinander durchgeführten

Experimente je einzeln zu besprechen; daher habe ich wegen Raumersparnis die Durchschnittszahl der gesamten Versuche in 3 Figuren zusammengestellt.

Ich habe zuerst die Ergebnisse der Wirkung der alleinigen Anwendung von verschiedenen Vitaminarten auf den Glykogenbestand des normalen Kaninchens in einer Figur zusammengestellt.

In der Figur 1 sind die Minuszahlen unter die Nullinie und die Pluszahlen über diese gestellt. Der Glykogenbestand des Kaninchens, das ohne jegliche Behandlung ruhig im Kasten blieb, vermindert sich in einer Stunde um 0.15%, und es zeigt sich, dass er mit der Zeit etwas abnimmt, aber ohne nennenswerte Schwankungen. Diese Schwankungen sind in den nachfolgenden Angaben immer als Kontrollzahl zum Vergleich in Betracht gezogen werden.

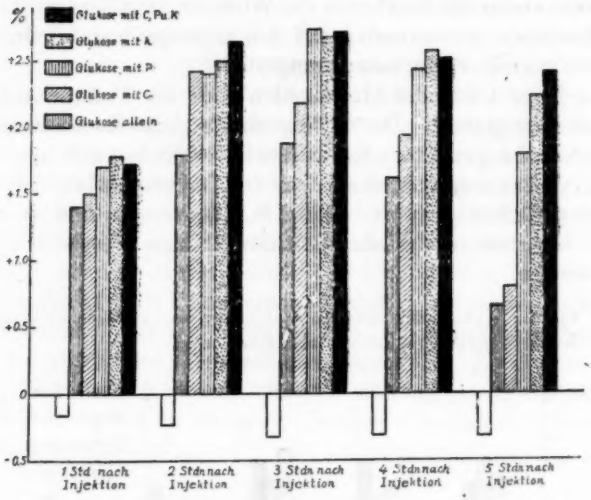
Fig. 1. Wirkung der alleinigen Anwendung der verschiedenen Vitamine auf den Glykogenbestand des normalen Kaninchens.




Bei der Anwendung von Vitamin C allein (100 mg pro kg) wird die Verminderung des Leberglykogens schon 1 Stunde nach der Injektion kompensiert (um 0.22%), in 2 und 3 Stunden je um 0.13 bzw. 0.42% wiederhergestellt, und in 5 Stunden ist die Wirkung des C-Vitamins schon vorüber. Bei der Anwendung von Vitamin P verläuft der Glykogenbestand ziemlich ähnlich wie bei C, aber beim Vitamin K ist das Leberglykogen im späteren Stadium etwa beibehalten, obwohl im früheren Stadium fast dasselbe Resultat beobachtet wurde.

In der Figur 2 sind die Ergebnisse der Glukose- und Vitaminversuche zusammengestellt. Bei der Darreichung von Glukoselösung allein wird das Leberglykogen schon in einer Stunde ziemlich stark gebildet (etwa 1.40%), in 2 Stunden um 1.79% und in 3 Stunden erreicht es die maximale Höhe, dann vermindert es sich allmählich und zwar in 5 Stunden um etwa 0.66%. Beim Zusatz von Vitamin C zur Glukoselösung beobachtet man noch mehr die fördernde Wirkung als bei Glukose allein, und zwar erreicht sie in 2 bzw. 3 Stunden die maximale Höhe, dann neigt sie allmählich zur Abnahme.

**Fig. 2. Wirkung der verschiedenen Vitamine auf die Neubildung des Leberglykogens durch intravenöse Infusion der Glukoselösung bei normalen Kaninchen.**




Das Zusetzen des Vitamins P bzw. K zur Glukose ruft ebenfalls eine Vermehrung des Glykogens hervor, und zwar in höherem Masse als beim C-Versuch. Charakteristisch ist aber, dass beim P- und K-Versuch die Zersetzung des Glykogens in späteren Stunden ziemlich aufgehalten wird.

Es wird deshalb sehr wahrscheinlich auf die fixierende Wirkung des betreffenden Vitamins zurückgeführt.

Nun ist es das Hauptaugenmerk darauf zu richten, wie sich das Glykogen der Kaninchenleber unter hoher Temperatur verhält und ausserdem wie die genannten Vitaminarten auf die Neubildung des Glykogens aus der Glukoselösung einzuwirken imstande sind.

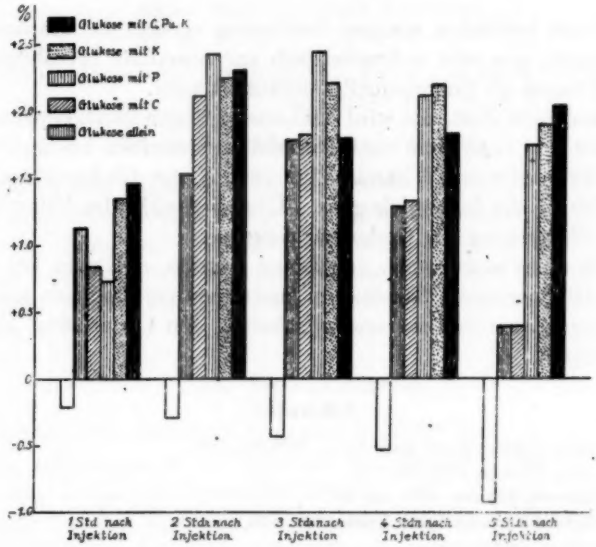
In der Literatur können wir verschiedene Berichte in bezug auf den Kohlehydratstoffwechsel der Leber unter besonders hoher Temperatur finden (Nozaka[7], Moszeik[8], Paton[9], Athanasiu[10], Inada[11], Iino[12], Niwa[13], Hasegawa[14], Imazu[15] und Ishimori[16]).

Obwohl die Versuchstiere und -bedingungen sehr verschieden waren, stimmten die Ergebnisse in der Abnahme des Glykogens fast überein, ausser beim letztgenannten Autor, der förderne Wirkung der Glykogenneubildung beobachtete.

Nun sieht man aus Fig. 3 eine ziemlich hochgradige Zersetztung des Leberglykogens unter Einwirkung hoher Temperatur (42°C), die besonders ausgeprägt ist in den 5 Stunden nach Einstellung in die Kasten.

Diese Zersetzung aber wird durch die alleinige Anwendung der Glukose ziemlich aufgehoben, in einem Masse, dass in 3 Stunden um 1.78% mehr als der präformierte Wert vorhanden war; doch es kann der Zersetzung durch Wärme nicht so lange trotzen, und nach 5 Stunden findet man nur etwa 0.39% mehr als zu Anfang.

Fig. 3. Wirkung der verschiedenen Vitamine auf die Neubildung des Leberglykogens durch intravenös infundierte Glukoselösung bei im Kasten 42°C erwärmten Kaninchen.




Ich habe zunächst Vitamin C (100 mg pro kg) zur Glukoselösung zugesetzt, um zu untersuchen, wie das Leberglykogen sich dazu verhält. Schon 2 Stunden nach der Injektion findet man eine beträchtliche Zunahme des Glykogens, nämlich eine Zunahme um 2.11%, d. h. sie ist um 0.58% höher als in der Glukose allein, jedoch um 0.31% geringer als bei demselben Versuch im normalen Zustand, was natürlich als ein Effekt der Wärme zu betrachten ist. Im weiteren Verlauf bis in der fünften Stunde vermindert sich das Leberglykogen allmählich aufs gleiche Niveau wie beim Zucker allein. Also kann man sagen, dass Vitamin C auf die Neubildung des Glykogens fördernd zu wirken imstande ist, aber man kann ihm wenig fixierende Wirkung zuschreiben.

Im Gegensatz dazu konstatiert man beim Gebrauch von Vitamin P bzw. K nicht nur fördernde, sondern auch fixierende Wirkung des neugebildeten Glykogens. Besonders bei der Anwendung einer Mischung von

allen 3 Vitaminen in der fünften Stunde findet man mehr Glykogen als in der 3. bzw. 4. Stunde. Es ist besonders hervorzuheben, dass die fixierende Wirkung sehr hervorstechend ist, während sonst unter der Einwirkung von Wärme in der 5. Stunde die Zersetzung des Glykogens am stärksten stattfindet.

## IV. Schluss.

Die oben erwähnten Ergebnisse kann man folgenderweise zusammenfassen.

1. Unter besonders warmer Bedingung verliert das Kaninchen das Leberglykogen, was sehr wahrscheinlich zur Annahme berechtigt, dass es vor allen Dingen als Energiequelle mobilisiert wird.

2. In diesem Zustande wird das Leberglykogen durch Glukoseinfusion kompensiert und ausserdem noch Neubildung desselben beobachtet.

3. Der Zusatz von Vitamin C, P bzw. K zur Glukoselösung fördert die Neubildung des Leberglykogens. Überdies wirkt das Vitamin P bzw. K auf die Zersetzung des Glykogens fixierend.

4. Es muss wohl warm empfohlen werden, dass man bei warmem Zustande Glukose und Vitaminarten zusammen verabreichen soll, um das Leberglykogen anzureichern und gleichzeitig den Organismus als ganzen zu bekräftigen.

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# Studies on the Detoxicating Hormone of the Liver (Yakriton).

## 103rd Report.

## A Working Hypothesis Concerning the Effect of Yakriton.

By

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In the present paper we desire to describe rather a working hypothesis than an experimental work, i.e. a working hypothesis concerning the effect of yakriton. Sato, one of the authors, was so fortunate that he was able to test his new hypothesis in a human case which had been unexpectedly offered him. Below will be described how he came to work out his new hypothesis.

### *The Eeffct of Yakriton.*

Yakriton is the detoxicating hormone of the liver. It can detoxicate ammonia; the amount of it is expressed by numbers of units for ammonia-detoxication (" Rabbit-Ammonia-Units "). But this is not the only specific effect of this hormone. It can also detoxicate phosphorus[1] quite specifically. It can detoxicate even different snake-poisons; Sato had not had the slightest expectation of this effect. He isolated yakriton[1] in 1925–1926 and since then he had been trying it for different poisons. But he never dreamed of trying it for snake venom. So it was with a great surprise that he read Ouchi's[3] clinical report concerning successful trial of this hormone for different snake-poisons. We are not going to state here different poisons to be detoxicated by yakriton, but such poisons and such diseases as for which yakriton had been reported as efficacious are shown in Table 1.

When Sato, one of the authors, was preparing the manuscript for the invitation address on yakriton at the 7th General Meeting of the Society for Diseases of Digestive Tractus " (=Shokakibyo-Gakkai) in 1940, he wanted to define this hormone as " normal keeping hormone " (Cf. 15th Report

TABLE I.

*Diseases and intoxications in which yakriton is used efficaciously.*

| Animal experimentation | Clinical |
|---|---|
| Different poisons or conditions to be detoxicated by yakriton | Poisoning of acridin dye† |
| Ammonium chloride | Anaphylaxis† |
| ,, carbonate | Anorexia† |
| Anaphylaxis | (Chronic) diarrhoea |
| Cantharides | Dengue icterus |
| Chloroform | Dühring's disease |
| Chromate | Dysentery |
| Dinitrophenol | Infantile dyspepsia and dyspepsia of young children |
| Dysentery toxin | Purulent diseases† |
| Hyperchloremia | Ekiri† |
| Hyperglycemia | Eklampsia† |
| Hunger | State of fatigue in old people |
| *Methyl alcohol | Nocturnal enuresis |
| Pepton shock | Epilepsia |
| Phenyl hydrazine | Hunger |
| Phenol | Hyperthyreoidism |
| Phosphorus | Icterus |
| *Salvarsan | Impetigo |
| *Santonine | Kakke |
| *Cobra venom | Spasmodic state† |
| Toluylene diamine | Paralysis |
| Uranium | "Otomimake"† |
| Urea | Periodical vomiting† |
| * The very first reports as to the efficacy of yakriton in the respective experiments were not published from our laboratory; these experiments were repeated later in our laboratory. | Phosphorus poisoning |
| | Pollakiuria |
| | Prontosil poisoning† |
| † The very first reports as to efficacy of yakriton in the respective diseases were published not from our Clinic, | Pruritus |
| | Salvarsan poisoning |
| | Scarlet fever |
| | Snake bite |
| | Uremia |
| | Urticaria |
| | Toxic condition (in various diseases) |
| | Weil's disease |

of Yakriton)[4] or normalizing hormone." But he (Sato) had thought of two exceptional instances. The first instance was the effect of yakriton on other hormones, and the second was regarding the effect on vitamins. It will act to counteract the effect of other hormones, while it will act to assist that of vitamins.

The above description was his brief summary concerning the effect of

yakriton on different poisons, hormones and vitamins. When he (Sato) had completed preparing the manuscript, he noticed the inconsistency between its effect on hormones and vitamins, and remembered that yakriton was used in experiments of avitaminosis or hypovitaminosis alone on one side, and in experiments with an excess alone of hormone on the other. He (Sato) immediately thought of the possibility:—In these experiments yakriton acted assistingly on vitamins, because there was too little vitamin. If it had been experimented with an excess of vitamin, yakriton might have counteracted the effect of it. Further, if the amount of other hormones used had been insufficient, then it must have acted assistingly upon them. If these two possibilities should turn out to be facts, yakriton would be simply defined as the normal keeping hormone—also with regard to its effect on vitamins and other hormones.

*Clinical Experiment.*

When Sato, one of the authors, thought of the two possibilities, as above mentioned, he was fortunate enough to try very soon one of the two in a case of a child who had come unexpectedly to us. She was an 11 year old girl suffering from hypothyreosis. At first we observed her hypothyreotic state—her inactive metabolism for about 10 days, during which only indifferent remedies had been given.

Now after the observation of the really inactive metabolism, it would have been quite natural that thyroid preparations or thyreoidin should be given to the hypothyreotic patient. In stead, we tried yakriton, which according to the results published so far would have counteracted the thyroid hormone and given thus an injurious effect to the child, whose metabolism would have become so much the more inactive. But if on the contrary yakriton be simply the normal keeping hormone according to the above explained possibility, it would exert a favourable influence on the patient, acting as if it had supplied more thyroid to her.

Now yakriton was actually injected once a day—in an amount of 1 R. A. U. (Rabbit-Ammonia-Unit.)—to the child after the observation of a period of really inactive metabolism for 10 days.

The result was quite favourable. The basal metabolism (here we thank heartily for the investigation of basal metabolism to Dr. Toraiwa in the Medical Department of Prof. Dr. Kato) increased markedly after yakriton injection. It was −10% on Aug. 31st a week prior to the injection, and was −5.8% on Sept. 10th a week after it (Cf. Table 2).

Only, this was our first experience; we dared not carry it too far. But then we wanted to observe also the effect of yakriton counteracting an excess of the same thyroid hormone in such a clinical experiment. Indeed

TABLE II.

*Basal metabolism in the hypothyreotic child (according to Krogh's method).*

| | Date | Use of yakriton and thyreoid | Basal metabolism |
|---|---|---|---|
| I | 24. VIII | With no yakriton and with no thyreoid | −10.0% |
| II | 7. IX | 8th day after yakriton injection | − 5.8 〃 |
| III | 12. 〃 | On the next day of use of yakriton and thyreoid | + 5.0 〃 |
| IV | 30. IX | 9th day of recommencement of thyreoid medication (without yakriton) | + 8.0 〃 |

we had tried yakriton in clinical cases of spontaneous hyperthyreoidism, but we had never tried experimental hyperthyreoidism in a human case. So we made use of a large amount of thyreoid (0.4 grms. on Sept. 10th and 0.2 grms., on the following day). This amount of thyreoid (0.6 grms. in all: three doses for 24 hours) was an excessive amount for the child, but yakriton administered simultaneously would, as we were convinced, counteract the excessive effect of thyreoid, so that no danger would result. The result was such as we had presumed. After this, no thyroid preparation was given till Sept. 22nd, though from this date on 0.1 grm. of thyreoid was given daily. The basal metabolism showed +8.0% on 9th day of daily administration of thyreoid (Cf. Table II). During the whole time the patient was very well and any unpleasant event did not occur at all.

This experiment showed us two important results above all. In the first place, yakriton was able to counteract an excessive (so large quantity of dose that we otherwise would not have ordered) does of thyreoid, but did not suppress the necessary normal effect of it. This first effect was not altogether against our expectation. The second result was rather astonishing: Yakriton acted favourably upon hypothyreotic state. It promoted the effect of thyreoid, which must have been secreted in only a small amount in the hypothyreotic body. The real effect of yakriton toward other hormones was for the first time brought to light by this clinical experiment.

### *The Effect of Yakriton Increasing Metabolism in Hypothyreotic State.*

As above discussed, yakriton acts assistingly upon thyroid hormone in hypothyreotic state, though there was naturally no thyreoid-like substance in yakriton itself. This effect was seen in increasing basal metabolism. It is further manifested in quantitative variation of urine ingredients.

Here we shall describe the variation of phosphate alone. Concerning the course of the patient and the variation of the other ingredients of urine, it will be reported in another paper (Cf. 104th Report of Yakriton)[7].

*The Variation of Urine Phosphates.*

Method: Bell and Doisy's[8] method for inorganic and total phosphates. 2.0 cc. of urine resp. 2.0 cc. of standard phosphate solution were diluted to 5 fold with water in a 25 cc. flask and filled with water up to mark. Then colorimetric determination.

Result: The daily excretion of inorganic and total phosphates is given in Table III. (Cf. Table III). The accompaning curve (Cf. figure) shows that during the yakriton period the phosphate excretion increased considerably, though this increase was still marked after the later administration of an excessive dose of thyreoid. But the phosphate excretion in this yakriton period was nearly equal to that in the next thyreoid period (with no yakriton( (Cf. Table IV). In the control period (without yakriton and without thyreoid) the average daily phosphate excretion was about 0.40 grms. and in the yakriton period it was ca. 0.65 grms; and this figure was not surpassed in the thyreoid period.

TABLE III.

*Variation of urine in the hypothyreotic child.*

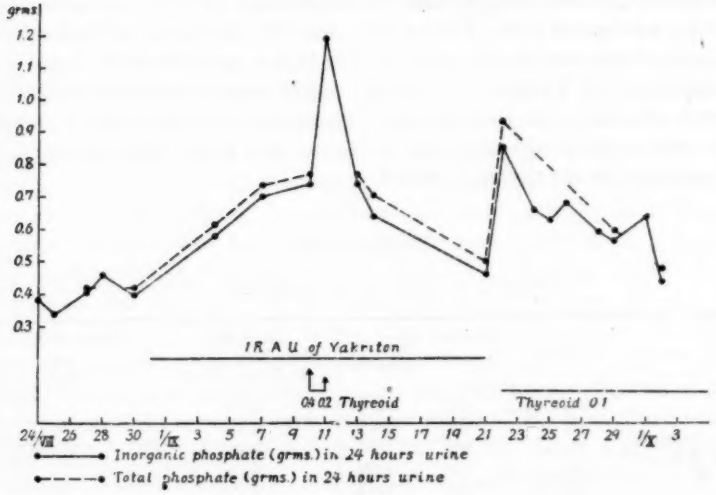
| | Date | Amount of urine (c.c.) | Specific gravity | Inorganic phosphate, grms. (24 hours urine) | Total-phosphate, grms. (24 hours urine) |
|---|---|---|---|---|---|
| No. yakriton; no thyreoid | 24.VIII | 900 | 1012 | 0.386 | |
| | 25. " | 800 | 1012 | 0.347 | |
| | 27. " | 900 | 1010 | 0.405 | 0.417 |
| | 28. " | 500 | 1022 | 0.406 | |
| | 30. " | 850 | 1012 | 0.395 | 0.410 |
| Yakriton alone | 4. IX | 1100 | 1014 | 0.580 | 0.617 |
| | 7. " | 1300 | | 0.708 | 0.739 |
| Yakriton and thyreoid | 10. " | 2000 | 1008 | 0.745 | 0.770 |
| | 11. " | 1600 | 1012 | 1.119 | |
| Yakriton alone | 13. " | 1000 | 1020 | 0.743 | 0.763 |
| | 14. " | 1150 | 1015 | 0.639 | 0.707 |
| | 21. " | 1000 | 1015 | 0.464 | 0.481 |
| Thyreoid alone | 22. " | 1600 | 1013 | 0.852 | 0.930 |
| | 24. " | 1200 | 1016 | 0.657 | |
| | 25. " | 1050 | 1017 | 0.626 | |
| | 26. " | 1100 | 1013 | 0.684 | |
| | 28. " | 1100 | 1012 | 0.599 | |
| | 29. " | 900 | 1015 | 0.563 | 0.596 |
| | 1. X | 1050 | 1015 | 0.640 | |
| | 2. " | 700 | 1018 | 0.443 | 0.455 |

TABLE IV.

*Daily average of phosphate excretion.*

| Experimental period | Inorganic phosphate in 24 hours urine (grms.) | Total phosphate in 24 hours urine (grms.) |
|---|---|---|
| Control (With no yakriton with no thyreoid) | 0,399 | 0,413 |
| Yakriton | 0,644 | 0,678 |
| Tyreoid (combined with yakriton) | 0,756 | — |
| Thyreoid | 0,633 | 0,660 |

Figure. Phosphate excretion in urine.




*Comment.*

First, a brief summary as to the hitherto reported relations between the thyroid gland and urine phosphate, between the thyroid gland and the liver, and further between the phosphate excretion and the liver, is reviewed here:—

1. Relation between the thyroid gland and urine phosphate.

Roos[1] and Falta[10] saw in animals a decreased excretion of phosphate after total thyreoidectomy. Aoike[11] proved the same thing. Yoshimatsu and Sakurada[12] saw an increase of the excretion of inorganic and total phosphates in a hypothyreotic child given a thyroid preparation.

Scholz[13] saw, in a case of a normal person, a more or less increased excretion of urine phosphate after taking thyroid tablets. Schittenhelm and Eisler[14] saw in a case of myxedema an increase of urine phosphate after thyroxin administration. Aoike[15] also proved the same result after continuous injection of thyroxin, and from the above mentioned works concerning thyroxin, he concluded that thyroxin raises (like the thyroid gland substance) the oxydative function of organism increasing metabolism. Uchida[16] saw, as Roos,[9] an increase of urine phosphate after thyroid gland medication, and saw the continuance of the increased excretion for a long time after even a suspension of its administration. Further he saw that the quotient N: $P_2O_5$ showed a marked reduction in cases fed on food with thyroid gland added, and he attributed the relative increase of phosphate excretion, as Schöndorff[17] did (we could not find in his work this explanation of Schöndorff cited in Uchida's and Aoike's[11] works) to an increasing decomposition of bodily protein resp. of tissue containing phosphates. Schöndorff[17] described: Feeding on thyroid gland exerts no influence upon protein metabolism, while the increase of nitrogen excretion at the beginning is the result of increasing excretion of urea and other nitrogen-containing extractive matters.

2. Relation between the thyroid gland and the liver.

According to Tsuji[18] an administration of thyroid gland will cause a hypertrophy of the liver of animals and thyreoidectomy an atrophy and degeneration of it. Kusakabe[19] states that bile secretion increased after the injection of thyroid extract, though it was unknown whether the increasing secretion was caused by stimulation of nerves controlling liver cells directly, or by stimulation of vasomotors of the liver.

3. Relation between phosphate excretion and the liver.

As Karasawa[20] and Hatakeyama[21] did previously, Sekito[22] saw an increase of urine phosphate by administration of bile acid and opined that, as this acid caused an increase of protein decomposition, an increase of phosphate excretion followed. Further he stated that the supply of bile acid decreased water excretion, increased calcium of blood and urine, increased excretion of urine phosphate and decreased protein metabolism, while he saw no influence of bile acid upon the excretion of chlorin, sodium, or potassium of urine.

In a forthcoming treatise (Cf. 104th Report of Yakriton)[7] it will be reported that in the present child case of hypothyreosis a marked increase of urine and of chlorin in urine etc. was caused by yakriton. This hormone has chemically no connection with bile acids.

Now the question: How yakriton, the detoxicating hormone of the liver, is able to raise basal metabolism and increase the excretion of urine

phosphate. This result will be best explained by its effect to act assistingly upon other hormones, thyroid hormone among the rest, in case of hormone deficiency. In the present case, the effect of yakriton is almost equal to that of thyreoid, which will be clearly seen from Table VII in the next report (Cf. 104th Report of yakriton)[7]. Yakriton acts antagonistically against an excess of hormone. But in the case of thyreoidin deficiency it acts assistingly upon the thyroid hormone. Yakriton always is and remains a normal keeping hormone. It will suppress hyperthyreoidism but it will make good for hypothyreoisis. This apparently peculiar action of yakriton will be probably seen in its effect toward vitamins. Only a few experiments (Cf. 163rd Report of Peroxidase Reaction)[23] and 68th Report of Peroxidase Reaction)[24] have been published concerning the action of yakriton upon vitamins. In these, yakriton was reported to assist the effect of a vitamin B; probably because the experiments were those of avitaminosis.

It is probable that yakriton will act antagonistically against an excess of vitamins.

*Remarks.*

1. As above stated, the other data of the experiment with regard to metabolism and clinical course of the present case will be reported in the 104th Report of yakriton.[7]

2. Remarks as to clinical use of yakriton in case of hormone of vitamin deficiency:—

In case of hormone or vitamin excess yakriton is used alone. But can we use yakriton alone in case of hormone or vitamin deficiency? In these cases, yakriton will act assistingly upon the effect of viatmins or other hormones. If the organisms concerned should not have any more store of a vitamin, yakriton can never develop any effect of that vitamin; that is a matter of course. So it is in practice advisable to give a small amount of vitamin or hormone concerned (as the case may be) besides yakriton.

3. In our present case we tried yakriton alone, without any use of thyroid or thyreoidin, for a week. Should its longer use have been harmful? If yakriton should act assistingly but sparingly upon the thyroid gland, then it may be used by itself for a long time. But if it (yakriton) should act to spur on the gland so that what little thyroid secrete might be exhausted soon, then an unfavourable result might occur. So, as above (Cf. Remark 2) mentioned, it is much safer in practical cases to use it together with a thyroid preparation.

## Conclusions.

1. Hitherto, yakriton, the detoxicating hormone of the liver, has

been defined as the "normal keeping hormone": There are two exceptions to this definition. One is: Yakriton counteracts other hormones and the other is: It assists vitamins.

2. Now our new definition of yakriton is: It is the normal keeping hormone. So it will counteract an excess of vitamins as well as other hormones, while it will act assistingly in cases of hormone deficiency as well as hypovitaminosis.

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# Studies on the Antibiotic Substances from Actinomyces.

## (1st Report)

### On the Isolation and Classification of Antibiotic Producing Actinomyces.

By

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(Received for publication, May 13, 1949)

(1) Many investigations were performed, in order to find a suitable conditions to isolate the strains of actinomyces from soils. It was elucidated, that asparagin glucose agar was a most suitable culture medium for this purpose because more actinomycetes and fewer bacteria were growing on it than on the other. It was further stated, that the distributions of actinomyces in soils were closely related to the kind of soils, to its pH and to the climate, at which the soils were collected.

(2) 1223 strains of actinomyces were isolated and cultivated on several kinds of fluid culture media and all culture filtrates were tested upon their inhibitory activities against *Staphylococcus aureus* (Terashima strain). By these methodes 157 (13%) strains were found to be antagonistic.

(3) So called "streak plate method" was, however, more sensitive and simple, and enabled us to form easily a specific "antibacterial spectrum." By this method it was possible to select 114 (44%) active strains under 261 strains.

(4) Representative strains of actinomyces were classified into several species by the characteristic properties on several differential culture media.

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# Uric Acid Content of Lactating Mothers' Urine and Arakawa's Reaction.

## Part 2.

## The Relation between Daily Variation of Uric Acid Content of their Urine and Arakawa's Reaction.

### 185th Report of the Peroxidase Reaction.

### (132nd Human Milk Study.)

By

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## *Introduction.*

In a preceding paper of mine[1] (184 th Report of the Peroxidase Reaction) I reported that there was a close relation between uric acid content of lactating mothers' urine and Arakawa's reaction* of their milk. In general, uric acid content in urine of mothers with Arakawa-negative milk was higher than that of mothers with Arakawa-positive milk. However it has not been proved yet that the excretion of uric acid per day is larger in the group of mothers with negative Arakawa's reaction than in the group of Arakawa-positive mothers.

In Part 1[1] of the present treatise I stated to the following effect:—

" The result of the animal experiments cited above concerns the whole day amount of uric acid excreted, while that of my own investigation was about the

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* Arakawa-positive may be used in two different senses. One of these is: Arakawa-positive in a biochemical sense. A sample of human milk is said to have become Arakawa-positive, when it becomes blue on the addition of Arakawa's reagent. Here it means that the sample is not negative to Arakawa's reaction. The other of these two senses is: Arakawa-positive in clinical sense. A sample of human milk is clinically Arakawa-positive only when it shows such a reaction as — or — in one minute of the addition of Arakawa's reagent. Another sample of human milk may be Arakawa-positive in the first described sense, but yet clinically negative.

uric acid concentration alone of one urine specimen voided in the morning. As to the whole day amount of the acid we have known nothing. But is it not probable in my cases that whole day urine was also of a higher uric acid content that in the group of Arakawa-negative mothers? Because in the group of Arakawa-positive mothers $\frac{40 \text{ mgrms.}}{100} \times 1000$† and in the group of Arakawa-negative mothers $\frac{70 \text{ mgrms.}}{100} \times 1000$† would represent an approximate amount or uric acid excreted during 24 hours.

But this way of calculation is not correct, even if we assume that the uric acid concentration in each group remained constant during one whole day, because the amount of the whole day urine is not equal, but different according to different groups of Arakawa's reaction. Arakawa-negative mothers who are in a state of B-avitaminosis* excrete, according to Umemura[2] (Cf. 155th Report of the Peroxidase Reaction), a smaller amount of urine during 24 hours than Arakawa-positive ones, they suffer from abnormal water-retention, though not from manifest edema. He stated that Arakawa-positive mothers excrete about 1150 cc. of urine, Arakawa-negative ones about 980 cc., and mothers of intermediate reaction about 1090 cc. respectively during 24 hours.

So the calculation shall be like the following:—

In the group of Arakawa-positive mothers,—

$$\frac{40 \text{ mgrms.}}{100} \times 1150 = 0.46 \text{ grms.}$$

or

$$\frac{40 \text{ mgrms.}}{100} \times 1200† = 0.48 \text{ grms.}$$

In the group of Arakawa-negative mothers,—

$$\frac{70 \text{ mgrms.}}{100} \times 980 = 0.69 \text{ grms.}$$

or

$$\frac{70 \text{ mgrms.}}{100} \times (800 \sim 900)† = 0.56 \sim 0.63 \text{ grms.}$$

In the group of mothers with intermediate reaction,—

$$\frac{55§ \text{ mgrms.}}{100} \times 1090 = 0.60 \text{ grms.}$$

or

$$\frac{55§ \text{ mgrms.}}{100} \times (900 \sim 1200)† = 0.50 \sim 0.66 \text{ grms.}$$

If the calculation above mentioned is right, then mothers negative to Arakawa's reaction will excrete the largest amount of uric acid per day and

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† 1000——assumed amount of whole day urine in c.c.

* $B_1$ is meant throughout the article.

† Rounded figures of urine amount according to Umemura are used.

§ $55 = \frac{50+60}{2}$

Arakawa-positive mothers the least amount of it, while the mothers of intermediate reaction will excrete an intermediate amount of it. We cannot decide whether this conclusion is right or not; we shall come upon this point in Part 2 of the present treatise."

It is almost impossible to collect whole day urine from healthy lactating mothers who visit our Dispensary for only a rather short time (a few hours). So I gave up such a plan and wanted to solve the problem by another means. Really healthy lactating mothers have constant Arakawa's reaction, viz. are of positive Arakawa's reaction generally, but many apparently healthy lactants show a variation in their Arakawa's reaction. I shall not enter into details about this matter; for which I only refer to the reports[3-6] published from our Laboratory. So I examined whole day urine of those lactating mothers whose sick breast-fed infants had been admitted to our Clinic, and studied the relation between the whole day amount of uric acid and the Arakawa reaction.

Arakawa's reaction and whole day amount of uric acid were daily examined, and the relation between them was studied. My own expectation was this: If during a space of time, say, during 5~6 days, Arakawa's reaction is negative, then uric acid excretion per day will be larger, and if then the relation has improved and remained of an intermediate reaction during a certain space of time, say, during 6~7 days, then the excretion per day will become smaller; or if Arakawa's reaction has become worse, then the uric acid excretion per day will increase. The present work is the result of such an investigation.

*Materials and Method of Investigation.*

Apparent healthy lactating mothers who fed their bread-fed patients during their admission to our University Hospital were objects of investigation.

In accordance with Höst's[7] and others' opinions, of all the factors which influence uric acid excretion, the diet is the most important. Furthermore it is an accepted opinion (Cf. Mathews)[8] that a diet which contains much nuclein, such as glandular organ like sweet-breads and liver, causes a remarkable increase in uric acid of human urine. So I paid attention daily to the menu from the Hospital-Kitchen and took care to keep off purine rich food from these mothers' meals.

In each case whole day urine from 8 a.m. to 8 a.m. was collected. I noted the total amount and the specific gravity of each urine, and then I estimated the uric acid content of the urine. Thus both the concentration and the total amount of uric acid for the whole day urine were known.

For the quantitative estimation of the uric acid in urine I employed

the method, described in Pincussen's book (Cf. Part 1[1] of the present treatise).

Milk was obtained each day 8 a.m. always before nursing[9] and examined with Arakawa's reagent. I classified Arakawa's reaction according to the intensity into 4 kinds: Positive, better intermediate, worse intermediate and negative (=completely or almost completely negative). The signs used to designate each reaction are the same as used in Part 1[1] of the present treatise (Cf. Table of signs).

Thus in this Part of the present treatise, the groups of different Arakawa's reaction were not those of lactating mothers, but the groups of days,—groups of days with the same intensity of Arakawa's reaction in each case, such as the group of days with positive reaction, of days with better intermediate, or days with completely negative reaction.

As to the urine, the amount of it and the specific gravity were also noted besides the amount of uric acid.

The results will be shown below.

### *Result of Investigation.*

Case 1 (Cf. Table 1, A and Table 1, B).

In this case there were 10 days of experiment. Basing upon the result of daily variations of Arakawa's reaction, I divided all the days into 2 groups, viz., 6 days belonging to the group of better intermediate reaction and 4 days belonging to the group of worse intermediate reaction. And the average results of these two groups were compared with each other (Table 1, B). The uric acid excretion was smaller (though slightly) in amount and concentration in the group of better intermediate reaction. The specific gravity was larger in the group of worse intermediate reaction. So the result came up to my expectation. The amounts of urine were equal; this was not necessarily against my expectation.

Case 2. (Cf. Table 2, A and Table 2, B).

In this case there were 13 days of experiment. Basing upon the result of daily variations of Arakawa's reaction, I divided all the days into 3 groups, viz., 5 days of better intermediate reaction, 6 days of worse intermediate reaction and one day of completely or almost completely negative reaction. And the average results of these three groups were compared with one another (Table 2, B). The better (=the stronger) the Arakawa reaction, are the content and amount of uric acid and the specific gravity the smaller, the amount of urine the larger. The result was thus as had been expected.

Case 3 (Cf. Table 3, A and Table 3, B).

In this case there were 7 days of experiment, and I divided all the days into 3 groups, viz., one day of worse intermediate reaction, 4 days of better intermediate reaction and 2 days of positive reaction. And the average results of these groups were compared with one another (Table 3, B). The better (=the

## Table I, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 1. Z. Sakurai, male, aged 13 months.
Diagnosis: Empyema pleurae sinistrum.
Age of mother, 32 years.

The intensity of AR is expressed in numbers, such as 10, 16 . . . (Cf. Y. Morikawi, Tohoku J. Exp. Med., 1943, **46**, 33).

| No. | Date | AR | | Ū content of urine | | Uirne (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 27. III | 10 | 24 | 35.45 | 0.319 | 900 | 1024 |
| 2 | 28. 〃 | 16 | 3 | 31.25 | 0.469 | 1500 | 1018 |
| 3 | 29. 〃 | 44 | 13 | 27.80 | 0.334 | 1200 | 1022 |
| 4 | 30. 〃 | 44 | 10 | 37.00 | 0.518 | 1400 | 1026 |
| 5 | 31. 〃 | 17 | 15 | 34.75 | 0.573 | 1650 | 1018 |
| 6 | 1. IV | 24 | 15 | 26.65 | 0.533 | 2000 | 1015 |
| 7 | 2. 〃 | 15 | 6 | 28.55 | 0.385 | 1350 | 1020 |
| 8 | 3. 〃 | 28 | 16 | 31.25 | 0.312 | 1000 | 1024 |
| 9 | 4. 〃 | 28 | 28 | 26.90 | 0.350 | 1300 | 1022 |
| 10 | 5. 〃 | 44 | 15 | 37.00 | 0.649 | 1700 | 1025 |

## Table I, B.

*Classification of the experiments in this Case, averag figures of uric acid content and amount of urine, according to the intensity of Arakawa's reaction.*

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 27. III~3.IV | Better intermediate | 31.32 | 0.432 | 1400 | 1020 |
| 29. III~5.IV | Worse intermediate | 32.17 | 0.463 | 1400 | 1024 |

## Table II, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 2. T. Aizawa, male, aged 8 months.
Diagnosis: Empyema pleurae dextrum.
Age of mother, 27 years.

| No. | Date | AR | | Ū content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 27.III | 7 | 13 | 33.30 | 0.266 | 800 | 1024 |
| 2 | 28. 〃 | 7 | 12 | 20.00 | 0.240 | 1200 | 1013 |
| 3 | 29. 〃 | 9 | 22 | 25.60 | 0.250 | 1000 | 1022 |
| 4 | 30. 〃 | 28 | 36 | 25.60 | 0.242 | 950 | 1024 |
| 5 | 31. 〃 | 12 | 46 | 23.50 | 0.259 | 1100 | 1022 |
| 6 | 1.IV | 25 | 48 | 30.00 | 0.360 | 1200 | 1015 |
| 7 | 2. 〃 | 38 | 48 | 37.80 | 0.302 | 800 | 1024 |
| 8 | 3. 〃 | 27 | 47 | 32.60 | 0.424 | 1300 | 1022 |
| 9 | 4. 〃 | 44 | 48 | 37.50 | 0.375 | 1000 | 1025 |
| 10 | 5. 〃 | 42 | 46 | 33.30 | 0.316 | 950 | 1026 |
| 11 | 6. 〃 | 20 | 36 | 35.75 | 0.358 | 1000 | 1024 |
| 12 | 7. 〃 | 4 | 15 | 27.50 | 0.413 | 1500 | 1025 |
| 13 | 8. 〃 | 8 | 23 | 28.00 | 0.336 | 1200 | 1026 |

## Table II, B.

(Cf. Table 1, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 2. III~6.IV | Better intermediate | 26.88 | 0.301 | 1140 | 1022 |
| 30. III~6.IV | Worse intermediate | 31.43 | 0.317 | 1017 | 1024 |
| 4. III | Completely or almost completely negative | 37.50 | 0.375 | 1000 | 1025 |

stronger) the Arakawa reaction, are the content and the amount of uric acid and the specific gravity the smaller, the amount of urine the larger. The result was thus as had been expected.

Case 4 (Cf. Table 4, A and Table 4, B).

In this case there were 8 days of experiment, and I divided all the days into 2 groups, viz., 5 days of worse intermediate reaction and 3 days of completely or almost completely negative reaction. And the average results of these two

## Table III, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 3. K. Endo, female, aged 6 months.
Diagnosis: Pneumonia.
Age of mother, 35 years.

| No. | Date | AR | | U content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 21. IV | 15 | 38 | 43.00 | 0.387 | 900 | 1020 |
| 2 | 22. 〃 | 28 | 3 | 37.75 | 0.378 | 1000 | 1018 |
| 3 | 23. 〃 | 2 | * | 27.75 | 0.361 | 1300 | 1015 |
| 4 | 24. 〃 | 2 | 16 | 30.25 | 0.272 | 900 | 1018 |
| 5 | 25. 〃 | 1 | 3 | 32.25 | 0.306 | 950 | 1018 |
| 6 | 26. 〃 | 2 | 8 | 33.75 | 0.338 | 1000 | 1015 |
| 7 | 27. 〃 | 1 | 1 | 24.00 | 0.288 | 1200 | 1013 |

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## Table IV, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 4. K. Itabashi, female, aged 26 months.
Diagnosis: Hernia diaphragmatica.
Age of mother, 29 years.

| No. | Date | AR | | U content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 22. IV | 46 | 48 | 60.75 | 0.668 | 1100 | 1024 |
| 2 | 23. 〃 | 42 | 48 | 60.75 | 0.851 | 1400 | 1020 |
| 3 | 24. 〃 | 48 | 48 | 54.25 | 0.814 | 1500 | 1025 |
| 4 | 25. 〃 | 47 | 48 | 67.75 | 0.881 | 1300 | 1030 |
| 5 | 26. 〃 | 43 | 48 | 57.00 | 0.684 | 1200 | 1025 |
| 6 | 27. 〃 | 36 | 47 | 65.50 | 0.524 | 800 | 1032 |
| 7 | 28. 〃 | † | * | 62.25 | 0.996 | 1600 | 1030 |
| 8 | 29. 〃 | 24 | 46 | 51.75 | 0.918 | 2400 | 1025 |

*= 1′ 2′ 3′ 4′ 5′
− − − − +
†= − + + + +

## Table III, B.

(Cf. Table 1, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Specific gravity |
| 21. IV | Worse intermediate | 43.00 | 0.387 | 900 | 1020 |
| 22. IV～26. IV | Better intermediate | 32.37 | 0.337 | 1050 | 1017 |
| 25. IV～27. IV | Positive | 28.13 | 0.297 | 1075 | 1016 |

## Table IV, B.

(Cf. Table, 1, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 23.IV～29.IV | Worse intermediate | 59.45 | 0.717 | 1360 | 1026 |
| 22.IV～25.IV | Completely or almost completely negative | 60.92 | 0.787 | 1333 | 1026 |

groups were compared with each other (Table 4, B).

The uric acid excretion was smaller in amount and concentration in the group of worse intermediate reaction. Besides, the amount of urine was larger in the group of worse intermediate reaction. So the result, too, came up to my expectation, though the specific gravity was equal.

Case 5 (Cf. Table 5, A and Table 5, B).

In this case there were 7 days of experiment, and I divided all the days into 2 groups, viz., 3 days of better intermediate reaction and 4 days of worse intermediate reaction. And the average results of these two groups were compared with each other (Table 5, B). The uric acid excretion was smaller in amount

## TABLE V, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 5. T. Mori, male, aged 7 months.
Diagnosis: Pneumonia.
Age of mother, 32 years.

| No. | Date | AR | | Ū content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 25. IV | 46 | 28 | 34.00 | 0.408 | 1200 | 1025 |
| 2 | 25. 〃 | 46 | 28 | 35.75 | 0.393 | 1100 | 1024 |
| 3 | 27. 〃 | 32 | 28 | 25.50 | 0.383 | 1500 | 1018 |
| 4 | 28. 〃 | 24 | 3 | 31.50 | 0.315 | 1000 | 1025 |
| 5 | 29. 〃 | 17 | 12 | 23.50 | 0.306 | 1300 | 1016 |
| 6 | 30. 〃 | 20 | 11 | 34.75 | 0.591 | 1700 | 1018 |
| 7 | 1. V | 7 | 36 | 33.75 | 0.506 | 1500 | 1025 |

## TABLE V, B.

(Cf. Table 1, B)

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 28. IV～30. IV | Better intermediate | 29.92 | 0.404 | 1333 | 1020 |
| 25. IV～1. V | Worse intermediate | 32.25 | 0.423 | 1325 | 1022 |

## TABLE VI, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 6. K. Watanabe, female, aged 21 months.
Diagnosis: Miliar tuberculosis.
Age of mother, 31 years.

| No. | Date | AR | | Ū content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 14. V | 7 | 7 | 49.00 | 0.735 | 1500 | 1022 |
| 2 | 15. 〃 | 13 | 13 | 62.25 | 0.436 | 700 | 1030 |
| 3 | 16. 〃 | * | * | 60.25 | 0.602 | 1000 | 1030 |
| 4 | 17. 〃 | 3 | 2 | 44.25 | 0.376 | 850 | 1025 |
| 5 | 18. 〃 | 2 | 2 | 55.25 | 0.718 | 1300 | 1026 |
| 6 | 19. 〃 | 2 | 1 | 45.75 | 0.686 | 1500 | 1020 |
| 7 | 20. 〃 | 3 | 1 | 53.00 | 0.504 | 950 | 1025 |
| 8 | 21. 〃 | 7 | 7 | 57.75 | 0.520 | 900 | 1030 |
| 9 | 22. 〃 | 8 | 2 | 69.00 | 0.587 | 850 | 1026 |
| 10 | 23. 〃 | 8 | 8 | 62.25 | 0.685 | 1100 | 1020 |
| 11 | 24. 〃 | 8 | 7 | 62.25 | 0.467 | 750 | 1030 |
| 12 | 25. 〃 | 12 | 2 | — | — | — | — |
| 13 | 26. 〃 | 7 | 1 | 76.50 | 0.536 | 700 | 1030 |
| 14 | 27. 〃 | 11 | 2 | 53.50 | 0.375 | 700 | 1015 |

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## TABLE VI, B.

(Cf. Table I, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 14.V～27.V | Better intermediate | 60.80 | 950 | 950 | 1025 |
| 17.V～20.V | Positive | 52.20 | 1100 | 1100 | 1025 |

and concentration in the group of better intermediate reaction. The amount of urine was larger, and the specific gravity was smaller in the group of better intermediate reaction. So the result came up to my expectation.

Case 6 (Cf. Table 6, A and Table 6, B).

In this case there were 14 days of experiment, and I divided all the days into 2 groups, viz., 10 days of better intermediate reaction and 3 days of positive reaction. And the average results of these two groups were compared with each other (Table 6, B). The uric acid excretion was smaller in amount and concentration in the group of positive reaction. The amount of urine was

## Table VII, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 7. T. Ono, female, aged 16 months.
Diagnosis: Pertussis pneumonia.
Aged of mother, 33 years.

| No. | Date | AR | | U content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 8.VIII | 44 | 48 | 90.75 | 1.361 | 1500 | 1025 |
| 2 | 9. 〃 | 44 | 48 | — | — | — | — |
| 3 | 10. 〃 | 39 | 39 | 95.25 | 1.148 | 1100 | 1026 |
| 4 | 11. 〃 | 39 | 39 | 93.00 | 1.023 | 1100 | 1026 |
| 5 | 12. 〃 | 46 | 31 | 78.00 | 0.780 | 1000 | 1020 |
| 6 | 13. 〃 | 40 | 19 | 66.25 | 0.994 | 1500 | 1024 |
| 7 | 14. 〃 | 48 | 48 | 90.75 | 1.225 | 1350 | 1020 |
| 8 | 15. 〃 | 48 | 48 | 93.00 | 1.023 | 1100 | 1024 |
| 9 | 16. 〃 | 44 | 47 | — | — | — | — |
| 10 | 17. 〃 | 28 | 48 | 63.00 | 0.567 | 900 | 1024 |
| 11 | 18. 〃 | 43 | 45 | 72.75 | 0.873 | 1200 | 1028 |
| 12 | 19. 〃 | 48 | 48 | 71.25 | 0.641 | 900 | 1025 |
| 13 | 20. 〃 | 36 | 22 | 81.75 | 1.226 | 1500 | 1030 |
| 14 | 21. 〃 | 42 | 38 | 63.00 | 0.665 | 1550 | 1020 |

## Table VIII, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 8. K. Oba, female, aged 9 months.
Diagnosis: Pulmonary tuberculosis.
Age of mother, 29 years.

| No. | Date | AR | | U content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 8.VIII | 27 | 27 | 61.50 | 0.308 | 500 | 1024 |
| 2 | 9. 〃 | 27 | 27 | — | — | — | — |
| 3 | 10. 〃 | 13 | 36 | 66.00 | 0.594 | 900 | 1018 |
| 4 | 11. 〃 | 28 | 39 | 84.00 | 0.336 | 400 | 1028 |
| 5 | 12. 〃 | 36 | 36 | 55.50 | 0.389 | 700 | 1026 |
| 6 | 13. 〃 | 46 | 48 | 59.25 | 0.415 | 700 | 1026 |
| 7 | 14. 〃 | 47 | 48 | 82.50 | 0.413 | 500 | 1028 |
| 8 | 15. 〃 | 47 | 48 | 84.50 | 0.507 | 600 | 1018 |

## Table VII, B.

(Cf. Table I, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 8.VIII～19.VIII | Completely or almost completely negative | 86.44 | 1.062 | 1225 | 1025 |
| 10.VIII～21.VIII | Worse intermediate | 76.63 | 0.910 | 1231 | 1025 |

## Table VIII, B.

(Cf. Table I, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 8.VIII～12.VIII | Worse intermediate | 66.75 | 0.408 | 625 | 1024 |
| 13.VIII～15.VIII | Completely or almost completely negative | 75.41 | 0.445 | 600 | 1024 |

larger, and the specific gravity was smaller in the group of positive reaction. So the result came up to my expectation.

Case 7 (Cf. Table 7, A and Table 7, B).

In this case there were 14 days of experiment, and I divided all the days into 2 groups, viz., 4 days of completely or almost completely negative reaction, and 8 days of worse intermediate reaction. And the average results of these two groups were compared with each other (Table 7, B). The uric acid excretion was smaller in amount and concentration in the group of worse intermediate

## Table IX, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 9. K. Maekawa, female, aged 6 months.
Diagnosis: Hydrocephalus and Lues cong.
Age of mother, 26 years.

| No. | Date | AR | | U content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 5.IX | 22 | 44 | 72.00 | 0.864 | 1200 | 1025 |
| 2 | 6. 〃 | * | 48 | — | — | — | — |
| 3 | 7. 〃 | 27 | 48 | 81.00 | 0.648 | 800 | 1025 |
| 4 | 8. 〃 | 47 | 24 | 109.75 | 0.878 | 800 | 1025 |
| 5 | 9. 〃 | 40 | 17 | 68.25 | 0.614 | 900 | 1024 |
| 6 | 10. 〃 | 30 | 36 | 59.25 | 0.593 | 1000 | 1020 |
| 7 | 11. 〃 | 13 | 36 | 53.25 | 0.479 | 900 | 1020 |
| 8 | 12. 〃 | 8 | 17 | 44.25 | 0.531 | 1200 | 1020 |
| 9 | 13. 〃 | 11 | 7 | — | — | — | — |
| 10 | 14. 〃 | 4 | 3 | 60.75 | 0.606 | 1000 | 1020 |
| 11 | 15. 〃 | 4 | 3 | 45.75 | 0.549 | 1200 | 1020 |

*=1′ 2′ 3′ 4′ 5′
+ ## ## ## ##

## Table X, A.

*Daily variation of Arakawa's reaction (=AR) and urine uric acid of lactating mother.*

Case 10, I. Doi, aged 12 months.
Diagnosis: Bronchopneumonia.
Age of mother, 25 years.

| No. | Date | AR | | U content of urine | | Urine (c.c.) per day | Spec. grav. |
|---|---|---|---|---|---|---|---|
| | | R | L | mgrms. per 100 c.c. | grms. per day | | |
| 1 | 18.XI | 44 | 48 | 85.55 | 0.941 | 1100 | 1032 |
| 2 | 19. 〃 | 48 | 7 | 53.25 | 0.533 | 1000 | 1022 |
| 3 | 20. 〃 | 43 | * | 68.75 | 0.963 | 1400 | 1022 |
| 4 | 21. 〃 | 44 | 48 | 87.00 | 1.218 | 1400 | 1022 |
| 5 | 22. 〃 | 47 | 48 | — | — | — | — |
| 6 | 23. 〃 | 39 | 10 | 49.50 | 0.792 | 1600 | 1028 |
| 7 | 24. 〃 | 38 | 2 | 69.25 | 0.623 | 900 | 1024 |
| 8 | 25. 〃 | 38 | * | 69.00 | 0.897 | 1300 | 1030 |
| 9 | 26. 〃 | 40 | 3 | 72.00 | 0.576 | 800 | 1030 |
| 10 | 27. 〃 | 22 | 3 | 57.00 | 0.912 | 1600 | 1020 |
| 11 | 28. 〃 | 12 | 8 | 66.75 | 1.001 | 1500 | 1028 |
| 12 | 29. 〃 | 23 | 12 | — | — | — | — |
| 13 | 30. 〃 | 30 | 15 | 37.50 | 0.563 | 1500 | 1018 |

*=1′ 2′ 3′ 4′ 5′
+ ## ## ## ##

## Table IX, B.

(Cf. Table I, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 5.IX～11.IX | Worse intermediate | 73.92 | 0.679 | 933 | 1030 |
| 12.IX～14.IX | Better intermediate | 52.50 | 0.569 | 1100 | 1020 |
| 15.IX | Positive | 45.75 | 0.549 | 1200 | 1020 |

## Table X, B.

(Cf. Table 10, B).

| Date | Arakawa's reaction | Urine (Average figures) | | | |
|---|---|---|---|---|---|
| | | mgrms. per 100 c.c. | grms. per 100 c.c. | Amount (c.c.) in 24 hours | Spec. grav. |
| 18.XI～21.XI | Completely or almost completely negative | 86.27 | 1.080 | 1250 | 1026 |
| 19.XI～25.XI | Worse intermediate | 61.38 | 0.796 | 1325 | 1026 |
| 24.XI～30.XI | Better intermediate | 60.50 | 0.670 | 1260 | 1024 |

reaction. Besides, the amount of urine was larger in this group, although the specific gravity was equal. So the result came up to my expectation.

Case 8 (Cf. Table 8, A and Table 8, B).

In this case there were 8 days of experiment, and I divided all the days into 2 groups, viz., 4 days of worse intermediate reaction and 3 days of completely or

almost completely negative reaction. And the average results of these two groups were compared with each other (Table 8, B).

The uric acid excretion was smaller in amount and concentration in the group of worse intermediate reaction. The amount of urine was larger in the group of worse intermediate reaction, although the specific gravity was equal. So the result came up to my expectation.

Case 9 (Cf. Table 9, A and Table 9, B).

In this case there were 11 days of experiment, and I divided all the days into 3 groups, viz., 6 days of worse intermediate reaction, 2 days of better intermediate reaction and 2 days of positive reaction. And the average results of these three groups were compared with one another (Table 9, B). The better (=the stronger) the Arakawa's reaction, are the content and amount of uric acid and the specific gravity the smaller, the amount of urine the larger. The result was thus as had been expected.

Case 10 (Cf. Table 10, A.and Table 10, B).

In this case there were 13 days of experiment, and I divided all the days into 3 groups, viz., 2 days of completely or almost completely negative reaction, 4 days of worse intermediate reaction and 5 days of better intermediate reaction. And the average results of these three groups were compared with one another (Table 10, B). The better (=the stronger) the Arakawa reaction, are the content and amount of uric acid and the specific gravity the smaller. But the amount of urine was the largest in the group of worse intermediate reaction; nevertheless, this was not necessarily against my expectation, because the amount of urine was the smallest in the group of completely or almost completely negative reaction.

### *Summary of Results.*

In all the 10 cases examined, the result was generally as had been expected in all the cases. My expectation was as follows: Uric acid excretion per day will be larger in the case of negative Arakawa's reaction than in the case of positive reaction. And this expectation of my own had been based upon the result of animal experiment reported in literature: B-avitaminotic animals excrete larger amount of uric acid (Cf. Part 1[1] of the present treatise).

Besides that expectation, I had another expectation based upon the result of Umemura[2]: Arakawa-negative mothers excrete a smaller amount of urine and mostly a urine of higher specific gravity. The result of the present experiment came up mostly to my expectation in this respect too.

## Discussion.

It is, as is introduced in a monograph (Mathews)[10], an accepted opinion at present that in man and the higher apes uric acid is the end-

product of purine metabolism, while in all the other mammals hitherto examined it is destroyed in the liver, giving rise to carbonic acid and allantoin. Further, it is generally recognized that in man uric acid arises from nuclein or other purine material ingested as food (exogenous origin of uric acid) or from the disintegrating cellular matter of the organism (endogenous origin).

As to the amount of uric acid in human urine there are plenty of accounts, but the figures of it in different reports are similar to one other. Mathews,[11] for instance, describes in his book that the excretion of uric acid varies from 0.3–1.2 grms. per day in the human adult, the average amount being about 0.6 grms. Scheube[12] reported of the daily uric acid output in urine of a Japanese to be 0.46 grms. on an average.

Concerning the factors which influence uric acid excretion opinions of different authors are almost in accord. Mathews[11] states that the amount of uric acid in urine varies with diet, state of health and individual idiosyncrasy.

As for the effect of diet, Mathews[10] says, a diet which contains much nuclein, such as glandular organ like sweet-breads and liver, causes an increase in uric acid excretion to 1 grm. and even 1.5 grms. per day. Hawk[13] writes in his book that a high purine diet may yield a daily uric acid output of 2 grms.

On the other hand, by cutting the nucleins completely off the food, or by starving, it is not possible to suppress the uric acid excretion entirely; according to Mathews[11] about 0.3–0.5 grms. of it per day are still excreted. It is generally believed to be due to endogenous source, and this endogenous uric acid itself varies in amount in different individuals.

Concerning the amount of endogenous uric acid, Siven[14] (1901), and Burian and Schur[15] (1901) claimed as a result of experimentation that it formed in any given period was fairly constant for each individual under normal conditions, and that it was entirely independent of the total amount of nitrogen eliminated. Folin[16] (1905), however, took exception to the statement of these investigators and claimed that, following a pronounced decrease in the amount of protein metabolized, the absolute quantity of uric acid was decreased, but that this decrease was relatively smaller than the decrease in the total nitrogen excretion and that the percent of the uric acid nitrogen, in terms of the total nitrogen, was therefore decidedly increased. Furthermore, Höst[7] (1919) said on a result of experimentation that with a fixed diet and under similar conditions the uric acid output in a few subjects was constant, while in the majority of those examined it was extremely irregular and showed variation from day to day up to 80 percent. According to him the endogenous uric acid output in 24 hours varied

between 0.27 and 0.99 grms; the last value was obtained in a normal person on an extensive bread diet.

In regard to the relation between vitamin B and uric acid excretion of human beings there is no conclusive opinion, so far as I know. But there are a few reports concerning the amount of uric acid in urine of man suffering from beriberi which is, as is well known, very closely related to avitaminosis-B.

It was quoted by Miura[17] that at the end of 19th century Vlaander reported the increase in uric acid excretion of beriberi cases, though I regret that Vlaander's original paper was not within my reach. Scheube[18] estimated uric acid of urine in some Japanese cases of beriberi and found in each case a decrease in its excretion; in one case of acute pernicious form he was scarcely able to find a trace of uric acid on the day before death.

Durham[19] stated that the purine bodies did not seem to be of great concern in a case of beriberi, though he recognized the decrease in uric acid output in this case. Teruuchi and Saiki[20] investigated the metabolism of a case of beriberi and found no marked change in uric acid excretion.

Sassa[21] also stated of urine of a slight case of beriberi, that the amount of uric acid in urine varied not remarkably in this disease, showing in his paper an analytic table (Cf. Table 11).

TABLE XI.

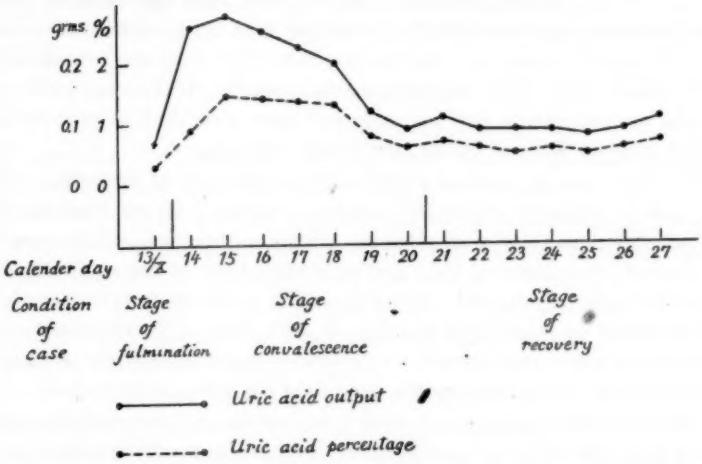
*Part of table of urine analysis of a slight case of beri-beri, compiled by Sassa.*[21]

| Date | Amount of urine in 24 hours c.c. | Specific gravity | Uric acid nitrogen grms. (%) | Condition |
|---|---|---|---|---|
| 24. V | 620 | 1.031 | 0.138 | Stage of exacerbation |
| 25. 〃 | 594 | 1.033 | 0.112 | |
| 26. 〃 | 658 | 1.033 | 0.121 | Stage of convalescence |
| 27. 〃 | 650 | 1.032 | 0.122 | |
| 28. 〃 | 574 | 1.034 | 0.117 | |
| 29. 〃 | 610 | 1.033 | 0.112 | Stage of recovery |
| 30. 〃 | 618 | 1.032 | 0.113 | |

Hasui[22] examined uric acid excretion in two cases of beriberi, and concluded that generally a slight decrease in uric acid excretion was found, and yet he admitted that uric acid output was increased when the total nitrogen was extremely increased.

But if one gives a glance at one of his figures (Cf. Fig. 1), one will be impressed with the thought that uric acid which had increased during the exacerbation decreased in the stage of improvement.

Fig. 1. Uric acid output and its percentage in case of beriberi, according to Hasui. (Case Yamazaki).




This led me to the possibility that uric acid excretion may increase in a case of beriberi and decrease only in an advanced stage of the disease. In animal experimentation of B-avitaminosis blood platelets will decrease in an advanced stage, but during a long period prior to the stage they will increase to an always higher figure. Sasaa's result may also be taken to mean rather that uric acid showed an inclination to increase in a stage of exacerbation and to decrease in the stage of improvement. Thus the clinical result is not necessarily in the sense of what the respective author concluded. Here we shall quote the result of animal experiments hitherto reported concerning uric acid excretion in B-avitaminosis.

In 1923 Adachi[23] reported* that dogs fed on B-avitaminotic diet had an increased excretion of uric acid in urine during their long timed B-carence and its later period especially. In 1924 Collazo[24] observed that, when dogs were fed on B-avitaminotic diet, uric acid content of their urine increased during their long time of B- hunger, although it decreased during the last time before death. In the same year Kudrjawzewa[25] also recognized that rabbits fed on diet deficient in vitamin B had an increased excretion of uric acid in urine. Further, in 1928 Lawrow and Matzko[26]

* These animal experiments were already introduced in Part I[1] of the present treatise.

reported that hens had an increased uric acid output in urine when they were fed on polished rice, i.e. on B-avitaminotic food.

Now, apparently healthy lactating mothers with Arakawa-negative milk are generally in a state of B-avitaminosis. This has been shown from different viewpoints by a number of papers from this Laboratory. In Part 1[1] of the present treatise I have shown that the urine of mothers with Arakawa-negative milk is of a larger uric acid content than that of Arakawa-positive mothers. And in this Part (Part 2) of the present treatise I have shown that, when lactating mothers secrete Arakawa-negative milk, the uric acid excretion per day is larger than then they secrete Arakawa-positive milk or milk of better Arakawa's reaction.

In other words, it is an indubitable evidence that lactating mothers with milk of negative Arakawa's reaction excrete a larger amount of uric acid per day than those with positive Arakawa's reaction. This conclusion will further be confirmed by a forthcoming paper of mine (187th Report of the Peroxidase Reaction). And this will be quite natural, because human milk negative to Arakawa's reaction is milk from a B-avitaminotic body. And it is, as above mentioned, a unanimous result of different authors that in animals fed on B-avitaminotic uric acid excretion is increased.

Here by way of appendix I want to reason about the result of my investigation from the point of pathological, anatomical and physiological view.

The accepted theory[27] of renal physiology in man at present assumes that urates are present in glomerular urine as are other ultrafiltrable substances and reabsorbed in part by the cells lining the tubulus. Therefore, if the cells lining the tubulus were at fault in any disease, there should be a disturbance of reabsorption of urates by tubulus and should be an increased excretion of urates in urine.

In this respect Coombs and Pecora[28] have lately published their observation that in the absence of severe renal impairment, all except 10 percent of the urates which were filtered through the glomeruli were reabsorbed by the tubulus, whereas with severe renal impairment reabsorption of urates by the tubulus was, they state, depressed and clearance tended to be maintained.

On the other hand, in beriberi disease, according to Yamagiwa,[29] there is a necrotic change of tubular epithelium, and according to Nagayo[30] there is a fatty degeneration of tubular epithelium, although there is no remarkable change in glomeruli.

Then, if I accept a very close relation between beriberi and B-avitaminosis, then an increased excretion of uric acid in urine in the case of Arakawa-negative mothers may be explained by disturbance of reabsorption by tubulus.

Here I desire to try to explain the higher concentration of uric acid in the case of Arakawa-negative mothers in urine specimens voided at one time. From this Part (Part 2) of the present treatise, it is highly probable that Arakawa-negative mothers excrete a larger amount of uric acid per day than Arakawa-positive ones. Now the former excrete a smaller amount of urine per day than the latter, then it is easily conceivable that, in general, in the former urine specimens should be rather remarkably concentrated in uric acid.

## Summary.

It was my first intention to know the relation between Arakawa's reaction and the uric acid excretion. Animal experimentation reported in literature showed unanimously that animals fed on B-avitaminotic food had an increased excretion of uric acid. Apparently healthy lactating mothers with milk negative to Arakawa's reaction are generally in a state of B-avitaminosis. If it is so, it will follow that Arakawa-negative mothers will excrete more uric acid per day than Arakawa-positive ones. This is the problem I wanted to solve.

As it is, however, very difficult to collect whole day urine of these healthy mothers, I wanted to solve the problem indirectly. Lactating mothers whose sick infants had been admitted to our Department were made objects of investigation, because it was less difficult to collect their whole day urine and they might, as I thought, change their Arakawa's reaction during their more or less long stay at our Hospital.

So I studied the relation of the change of Arakawa's reaction and the amount of uric acid excreted in 10 lactating mothers. When their Arakawa's reaction changed from worse to better, they were considered to have changed from worse to better with regard to their B-avitaminotic state. (This consideration was the natural result of a number of investigations from our Laboratory).

The result was: 1. When Arakawa's reaction was positive, the amount of uric acid excreted was the smallest, and 2. in the case of completely or almost completely negative Arakawa's reaction, its amount was the largest. 3. And in the case of intermediate reactions, its amount was of intermediate values.

It was thus highly probable that apparently healthy Arakawa-negative mothers, who are in a state of B-avitaminosis, would excrete a larger amount of uric acid per day than Arakawa-positive mothers.

Besides, the following result was seen. Umemura[2] of our Laboratory showed that Arakawa-positive mothers generally excreted a larger amount of urine per day than Arakawa-negative ones, and that specific gravity of

urine was generally smaller in the former than in the latter.

In my own investigation, when Arakawa's reaction improved, lactating mothers excreted generally a larger amount of urine per day and the specific gravity of their urine became smaller generally.

What has been stated in the Summary will further be confirmed by the result of my own, which will be reported under the title: Influence of Vitamin B Administration upon Urinary Uric Acid Content and Arakawa's Reaction of Lactating Mothers.

## Conclusions.

In 10 cases of lactating women of different Arakawa's reaction, who showed an improvement or exacerbation of the reaction during my observation, it was known that the amount of uric acid excreted per day was larger in the period of weaker Arakawa's reaction than in the period of stronger reaction. That is, when lactating women are more B-avitaminotic, they excrete a larger amount of uric acid per day than when they are less B-avitaminotic. This result is in conformity with the result of animal experimentation reported in literature that, when animals are fed on B-avitaminotic food, they excrete a larger amount of uric acid.

It is highly probable that lactating mothers with negative Arakawa's reaction excrete a larger amount of uric acid per day than Arakawa-positive mothers, and that Arakawa-negative mothers are generally in a state of B-avitaminosis, however healthy they may appear.

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Prof. Akira Sato.

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# Studies on the Antibiotic Substances from Actinomyces.

## (2nd Report)

### On the classification of Antibiotic-Producing Actinomyces by the Streak plate Method (So-called "Primary Screening").

By

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360 strains of active actinomyces were selected by "streak plate method" and classified by their antibacterial spectrums into following 4 groups;

1. group: Inhibitive upon Gram positive, Gram nagative and acid fast organisms.....201 strains (56%).....
2. group: Inhibitive upon Gram positive and acid fast organisms.... ..... 130 strains (36%)
3. group: Inhibitive upon Gram positive organisms only..... .....14 strains (4%)..
4. group: Inhibitive upon acid fast organisms only..... .....15 strains (4%)..

The strains of actinomyces of each group were divided further into several subgroups by their special antibacterial spectrums.

The authors named this method as "Primary screening," which is a starting point of our systematic investigation on the antibiotics of actinomyces.

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# Biochemical Studies on Carbohydrates.

## CV. Structural Study of A-specific Carbohydrate from the Mucin of Pig Stomach-mucus.

First Paper.

By

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(Received for publication, March 5, 1947)

From the complex carbohydrate in the heading acetolysis, deacetylation and following fractionation by means of organic solvents gave an acetylglucosamine galactoside which makes the unit structure of the mother substance. The chemical and physical properties of this disaccharide will be seen in the experimental part.

### Experimental.

### Preparation of the Group Specific Polysaccharide Employed as a Material.

The principles of Kobayasi-Tiba[1] and Satoh[2] methods were combined.

*First stage.* 85 gm. of pig stomach mucus were stood with 170 cc. of 1.5 n. NaOH solution at room temperature (10–17°C) for one day and to the digestate was added glacial acetic acid to pH 5.0 under cooling. Centrifuged. The supernatant fluid and the washing (a dilute acetic acid at a similar pH was used) of the centrifugate were united, dialyzed (cellophane membrane) against running water until neutral (three days were required), and filtered through a folded paper to remove some material which had separated. It was then evaporated under reduced pressure to 8 cc., 85 cc. of 94% alcohol added, and the precipitates washed with two 8 cc. portions of 96% alcohol and dried in a desiccator over $H_2SO_4$. The obtained powder weighed 2.1 gm.

*Second stage.* The substance was at first made into a thick dough with a little water, and after increasing water to 35 cc., the insoluble part was centrifuged off. The supernatant liquid was treated further as the

alkaline digestate of the mucus:— a) Acidified, centrifuged repeatedly, and when made water-clear, subjected to dialysis; b) then filtered, condensed and precipitated by the use of alcohol. The precipitates were washed with alcohol and dried. Crop, 1.1 gm.

*Third stage.* The product was agitated thoroughly with about 20 cc. of glycol, and to the transparent supernatant liquid after prolonged centrifugation were added 10 volumes of glacial acetic acid (96%). The deposit was centrifuged and washed with four 5 cc. portions of 95% alcohol and dried. Crop, 0.81 gm.

*Fourth stage.* This substance was dissolved in 40 cc. of water and shaken with 4 cc. of 12.5% $CuSO_4$ solution and 8 cc. of 10% NaOH solution. The centrifugate was washed with 8 cc. of 2% NaOH solution four times and dissolved in 4 cc. of 10% HCl solution, followingly the solution was precipitated with 60 cc. of 95% alcohol and the precipitates washed repeatedly with 95% alcohol, till free from HCl, and dried. The processes were repeated once more, when no biuret reaction was given. Crop, 0.61 gm.

*Fifth stage.* Electrodialysis. 0.61 gm. was adapted as 1% solution to electrodialysis (Pauli apparatus, collodione membrane, 200 volts). On the fourth day, the new changes of water in the outer compartments showed no pH shift. The precipitates, which appeared in the meanwhile, were centrifuged off and the clear supernant liquid distilled under reduced pressure to about 3 cc. The substance thrown down with 40 cc. of 95% alcohol was washed twice with 10 cc. of 95% alcohol and dried in a desiccator over $H_2SO_4$. A powder weighing 0.51 gm. was yielded.

## Acetolysis.

The procedure of Bergmann and others[3] was applied:—

3 gm. of the carbohydrate prepared as above and perfectly dried were taken in an Erlenmeyer flask and placed in a freezing mixture. To it was added a mixture[4] of 15 cc. acetic anhydride and 2.1 cc. sulfuric acid (99.5%), cooled as well, in small portions and the stoppered vessel (ground joint) was kept in a dark room (15–20°C) for two days with occasional shaking (the substance turned yellow), and subsequently in a thermostat at 52–53°C. In 10 hours about 2/3 of the substance dissolved, giving a dark red solution. The suspension was cooled and centrifuged to again subject the undissolved part to the same treatment. The supernatant liquid was poured into 90 cc. of ice-cooled water containing 15 gm. of crystalline sodium acetate under agitation, whereupon red-brown, oily deposit came out, and sodium carbonate at first and afterwards sodium bicarbonate added to neutral. The oil changed thick-pasty by the

neutralization, but without separating it off, the mixture was shaken with chloroform repeatedly (with one 50 cc. portion and two 30 cc. portions). On the other hand, the part of the material, which was re-acetolyzed, gave a complete solution. This was also diluted, neutralized and extracted with chloroform. The chloroform extracts from the both solutions were united altogether and after washing with 100 cc. of water twice, dried by means of $CaCl_2$ (5 gm.) in a dark room. $CaCl_2$ was filtered off on a creased filter and washed with chloroform, then the filtrate and washings combined were condensed at a low temperature (in vacuo, $<30°C$). The syrupy residue was taken up in 5 cc. of methanol. Since a few flocculent precipitates came out of the solution on standing in an ice chest, these were separated off on a Hirsch funnel and washed with methanol (3 cc., three times), and the filtrate and washings were evaporated together similar to before. The remainder dried in a vacuum desiccator was 3.0 gm. and proved clearly soluble in methanol (a red solution was given).

### Deacetylation of the Cleavage Products and Following Fractionation.

The whole of the product above was deacetylated by shaking with 135 cc. of 1/5 n. KOH solution for 4 hours (room temperature, 15°C). The red solution obtained was neutralized with 1 n. $H_2SO_4$ solution (2.5 cc. were used) and distilled in vacuo ($<38°C$) to a syrup. This was again evaporated twice with 50 cc. of methanol and the completely dried remainder was boiled three times with 50 cc. of methanol under reflux. The extracts were filtered hot and concentrated[5] as above ($<35°C$) to a dark red syrup and treated with boiling absolute alcohol (two 30 cc. and one 10 cc. portions of the alcohol were used). Flocculi separated from the extract, when cold, and increased on raising the volume of the solvent. Therefore cold absolute alcohol (100 cc. in total) was supplemented, until no more precipitation occurred. The centrifuged supernatant liquid was distilled (under reduced pressure, $<40°C$) to dryness, and the amorphous residue was taken up in 100 cc. of cold methanol (the substance dissolved well), decolorized with activated charcoal (2 gm. were employed) and after distilling off the solvent, extracted with cold absolute alcohol repeatedly. When evaporated and dried, the extract left a substance which weighed even 3.2 gm. and largely consisted of ash. 20 cc. of butanol dissolved it almost entirely but precipitation happened successively by further addition in portions of butanol up to 200 cc. The precipitates were collected by centrifugation and dissolved in 25 cc. of 70% ethanol. A small part undissolved was separated off on a Büchner funnel and washed with 70% alcohol (two 10 cc. portions) and the filtrate and washings were evaporated together under reduced pressure ($<40°C$) to a syrup (red-

coloured). It[7] was then dissolved in 10 cc. of 70% ethanol and 50 cc. of absolute ethanol added. Some precipitates coming out on addition of 20 cc. ether were discarded and the clear mother liquid was precipitated most possibly with ether (500 cc.). The flocculent precipitates here were collected by centrifugation and dried up (in vacuo over $H_2SO_4$)—550 mgm.

In order to eliminate ash, which was largely potassium acetate, the following method was used. The substance was dissolved in 10 cc. of water, and after cautious addition of 10% $H_2SO_4$ to pH 1.2, extracted with ether continuously overnight. It was then neutralized with the saturated $Ba(OH)_2$, distilled (<35°C), without antecedent removal of $BaSO_4$-precipitates, nearly to dryness and extracted with hot absolute methanol (four 30 cc. portions). Condensation and drying of the extracts gave 490 mgm. of yellowish white very hygroscopic powder. This was the disaccharide in view as will be demonstrated by analytical figures.

## Physical and Chemical Properties of the Starting Polysaccharide and the Product.

For the purpose of analytical and other quantitative determinations the substances were dried constant in vacuo over $P_2O_5$ at room temperature.

### 1) The group-specific carbohydrate for acetolysis.

Biuret and ninhydrin reactions and the tests for methylpentose (Rosenthaler's and Oshima-Tollens' methods) were negative. Molisch, intensively positive. Quantitative figures given were: N (micro Kjeldahl), 4.12%; acetyl (a modification of Friedrich, Rapoport and Sternberg's method by Suzuki), 10.77%; glucosamine (Hamasato and Akakura method), 44.52%; galactose, 45.16% (the indole reaction of Dische, 45.16%; the orcinol reaction of Tillmans and Philippi simplified by Ozaki, 45.16%); ash (micro analysis), 0.03%. It exhibited anti-isoagglutinative reaction to A erythrocytes at $1:10^6$ dilution.

### 2) The final product (acetylglucosamine-galactoside).

A red dye developed by *p*-dimethylaminobenzaldehyde, when the substance had been heated in advance with alkali (Osaki and Turumi procedure), and strong Molisch reaction was also given. It had not the serological potency above at a dilution of 1: 100. Decomposition point, 175–185° (s. below). Molecular weight (Rast method), 386.65 (calc., 383.35). Rotation of light in water: it had the initial $[D]_{\infty}^{t}$ of +66.38° (3 minutes after dissolution) and equilibrium rotation of +57.81° (after 12 hours from dissolution). N (micro Dumas), 3.61% (calc., 3.65%); acetyl, 11.21% (calc., 11.23%); galactose, 47.13% by the indole method,

47.03% by the orcinol method, in averages 47.08% (calc., 47.0%); iodine use as glucose (Macleod and Robison), 49.54% (calc., 47.00%); ash, 0.1%. It tasted sweet.

Details will be appended regarding the molecular weight, optical rotatory power and melting point :—

Molecular weight. 0.557 mgm. of the substance was mixed with 4.943 mgm. of campfor. The depression of melting point was 11.6 degrees. M. w.$=\frac{1000\times0.557\times40}{4.943\times11.6}=386.65$.

Optical rotation. 22.40 mgm. of the substance were dissolved in water at 4°C and made up accurately with water to 1 cc.

| Duration of time after dissolution (min.) | $\alpha$ (degree) | $[D]_a^4=\frac{\alpha\times100}{1\times2.240}$ (degree) |
|---|---|---|
| 3 | +1.487 | +66.38 |
| 5 | +1.437 | +64.15 |
| 10 | +1.397 | +62.37 |
| 15 | +1.379 | +61.56 |
| 20 | +1.367 | +61.03 |
| 25 | +1.357 | +60.58 |
| 30 | +1.351 | +60.31 |
| 40 | +1.339 | +59.78 |
| 50 | +1.333 | +59.51 |
| 60 | +1.331 | +59.42 |
| 60×2 | +1.320 | +58.93 |
| 60×3 | +1.313 | +58.62 |
| 60×12 | +1.295 | +57.81 |
| 60×24 | +1.295 | +57.81 |

Melting point. It began to swell at some 98°C to contract again at 135°C and upwards, and melted very slowly at 175–185°C under decomposition.

Through the Grant Committee for Scientific Researches of the National Research Council was given from the Education Department a grant which enabled us to carry out this investigation. H. Masamune.

## Bibliography and Notes.

1) Tiba, Tohoku J. Exp. Med., 1949, **50**, 281.
2) Satoh, Tohoku J. Exp. Med., in press.
3) Bergmann, Zervas and Silberkweit, Ber. deutsch. chem. Ges., 1931, **64**, 2436.
4) Acetic anhydride and sulfuric acid were both cooled before they were mixed together.
5) On standing in air the filtrates became turbid but were immediately subjected to distillation.
6) They were insoluble in absolute alcohol.
7) Insoluble in butanol.

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# A One-dimensional Diffusion Method of Assaying Streptomycin.

By

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We developed a suitable method for assaying streptomycin as follows and are now using the method for the preparation of streptomycin and other similar antibiotics and for the estimation of streptomycin in body fluids.

0.5 cc. of streptomycin solutions of various concs. (2.5/8, 2.5/4, 2.5/2, 2.5, 5, 10 and 20 units in a buffered diluent, pH 8.0) were superimposed on 2.5 cc. of inoculated agar medium (dehydrated nutrient broth 0.5 g, Sod. nitrate 0.02 g, Agar 0.7 g, Water 100 cc., added with 0.5 cc. of 24 hours broth culture of *St. aureus* in a melted state at 45°C and divided into small test tubes). After 12 hours incubation at 30°C the lengths of inhibited zones were measured and plotted on graph paper, with the numbers of concs. of streptomycin in logarithmic scale on abscissa. The following formula was determined experimentally:

$y(x)=G(1-e^{-r(x-a)})$, where $y(x)$ is the length of inhibition zone at log dose $x$ and $G$, $r$ and $a$ are their parameters. But between 2.5/4 to 10 units the inhibition curve was nearly a straight line. So for the estimation, the formula $y=ax+b$ was used, where $a$ and $b$ were two parameters.

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# Biochemical Studies on Carbohydrates.

## CVI. On Seromucoid.

(A Supplementary Note to XVIII[1], LXVI[2] and LXXX[3] Informations).

By

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(Received for publication, March 19, 1947)

Hewitt[4] put forward the presence in blood serum of a non-heat-coagulable glycidamin[5] "seroglycoid," which possesses about 10% carbohydrate in the molecule, and regarded seromucoid (Zanetti[6]) as a decomposition product of seroglycoid due to heating or by the action of serum proteases, for the reason that the latter is cleft very readily by pepsin, giving on very brief (5 minutes) digestion a 24% carbohydrate-containing product, and besides because he failed to detect the former.

Subsequently, however, Rimington[7] said in 1940 that, in purified seromucoid, carbohydrate accounts for not more than 10.7% and laid stress upon the close resemblance of his preparation in many respects with "seroglycoid."

According to recent investigations, denaturation appears to be an alteration of the structure in the protein molecule but not an actual cleavage[8]. High temperature or alcohol most probably causes the rearrangement of linkages also in glycidamins, whose amino acid-grouping belongs to the globulin class or is of an allied nature[9], so that the non-coagulability of water-soluble mucoids is assumed as maintained by a large carbohydrate moiety. In all well-established water-soluble mucoids such as those from the white and the yolk[10] of hen eggs and the mucosa of bull stomach[11], the carbohydrate content exceeds 20%, and we wonder if a glycidamin with only some 10% carbohydrate, whether seroglycoid or Rimington's seromucoid, does not loose solubility even if partially, when kept long in dry status after having been treated with alcohol. In fact, our crude mucoid, which involved a similar amount of carbohydrate, became, as will be mentioned below, more and more difficultly soluble with time on standing. Incidentally it should be added that the marked cleavability

quoted of seroglycoid suggests its nature of associated complex and furthermore experimentation by the use of a gastric enzyme can not furnish a persuasive argument for the view of the author on the occurrence of seromucoid of Zanetti.

From these considerations, we schemed to see if a permanent soluble protein with an abundant carbohydrate like ovomucoid is actually obtainable. The whole procedure applied was essentially similar to Rimington's[7], but the alcoholic fractioning of the initial crude seromucoid was preceded by treatment with boiling alcohol. Boiling alcohol proved not to decompose ovomucoid and hence it was used to quickly complete the elimination of admixed coagulable proteins. Analyses showed that, in carbohydrate content, the unfractioned material was not far removed from the mucoids of Rimington and of Ozaki, although we disagree with these authors in connection with its quantitative composition. Only the fraction, which remained in solution at the alcohol concentration of 60% and precipitated on increasing alcohol to 70% ($Fr_{70}$), dissolved in water clearly but all the other, including the one recovered at a still higher alcohol concentration, changed partially insoluble on standing. In harmony therewith $Fr_{70}$ was richest in carbohydrate and the percentage of the latter resembled the value of Bywaters[12] and that reported by Rimington in 1933[13]. This fractions was perfectly soluble even after months and may be looked at as real seromucoid. In our case was not obtained any fraction of peptone-like bodies with carbohydrate amounting even to 43%, as Rimington had separated.

In Tables $II_a$–$II_b$ are assembled the quantitative data. The methods of analysis were those heretofore employed in this laboratory. To start with, the figures of our pure seromucoid ($Fr_{70}$) will be discussed. The glucosamine amount reached two thirds of the corresponding amount of total non-amino sugars (the sum of equimolecular mannose and galactose), whereas Rimington[7] found the ratio 1:2. That Hamasato and Akakura method[14] for hexosamine determination gives accurate and reproducible results has been already demonstrated on a number of glycidamins and glycoproteins as well as their polysaccharides liberated. Acetyl was present in excess over the equivalent to the amino sugar as had been expected from the observation of Osaki and Turumi[15] that this mucoid[16] gives with Ehrlich *p*-dimethylaminobenzaldehyde reagent not only the specific reaction taking place after treatment with hot alkali but also the immediate, because to the direct reaction-giving structure of the hexosamine derivative are attached two acetyl rests which are liberated when heated with acid (Hiyama[17]). The same interrelations were seen also with respect to the original crude mucoid and the fraction left unextracted by hot water. These findings indicate the integrity of the carbohydrate that

H. Suzuki[2)3)] separated from crude seromucoid and had an excess acetyl.

## Experimental.

### Preparation and Fractionation of Crude Seromucoid.

2 *l.* of serum quickly separated by centrifugation of fresh defibrinated ox blood (7 *l.*) were diluted with 2 volumes of water. The diluted serum was acidified to pH 4.7 with 80 cc. of 30% acetic acid, boiled for 1 hour and soon NaCl powder added to ensure maximum precipitation. After standing for a while at room temperature, the coagula were filtered off on a Büchner funnel and the clear filtrate distilled under reduced pressure (50°C) to 1 *l.* A small precipitate appeared. Though the filtrate from it was opalescent, this was dialyzed as such against running tap water (thymol was used as a preservative). The opalescence-giving proteins were thereby thrown down. The fluid removed from the precipitate was condensed, similar to above, to 100 cc. Turbidity occurred again here. To the centrifuged supernatant liquid which was opaque and coloured brownish yellow, were added 4 volumes of 94% alcohol and the deposit washed with alcohol and ether and dried in a desiccator. About 0.5 gm. of grayish powder was yielded.

From the crude products thus obtained 1 gm. lots were boiled with 75 cc. of 95% alcohol under reflux for 10 hours and then, when cold, separated, washed with two 30 cc. portions of 95% alcohol and 30 cc. of ether and placed in a vacuum desiccator ($H_2SO_4$). The dried substance (980 mgm.) was kneaded with a little water, the volume of water raised to 100 cc. and the mixture boiled for 2 hours under agitation, water being compensated from time to time. Cooled and filtered. The filtrate (97 cc.) was yellowish and transparent. After adding 1.5 gm. of NaCl, its fractional precipitation with alcohol (95%) was exerted as illustrated in Table I. $Fr_{80}$ was obtained by taking up 100 mgm. of $Fr_{alc.-et.}$ in 5 cc. of water,

Table I.

Alcoholic Fractionation of the Extract of Crude Serum Mucoid.

| Fraction | Volume of solution before adding or increasing alcohol c.c. | Concrement of alcohol cc. | Final conc. of alcohol % | Amount of precipitates mgm. | Solubility in water |
|---|---|---|---|---|---|
| $Fr_{20}$ | 97 | 24.57 | 20 | | |
| $Fr_{30}$ | 119 | 18.3 | 30 | 58.4 | partially |
| $Fr_{40}$ | 139 | 24.9 | 40 | ($Fr_{20}$–$Fr_{50}$) | insoluble |
| $Fr_{50}$ | 155 | 34.44 | 50 | | |
| $Fr_{60}$ | 184 | 52.57 | 60 | 105.7 | almost soluble |
| $Fr_{70}$ | 231 | 92.4 | 70 | 67.8 | clearly " |
| $Fr_{alc.+et.}$ | 310 | 1550+ ether(310 cc.) | | 143.1 | partially insoluble |
| $Fr_{80}$ | | | 80 | | almost soluble |

centrifuging off the insoluble part and finally adding 95% alcohol to the supernatant fluid (4.2 cc.) up to 80%. Crop, 69 mgm. All the fractions were dried in a desiccator over $H_2SO_4$.

TABLE IIa.

Analysis of the Fractions of Crude Serum Mucoid.

The figures express the content in percentages of the material.

| Analysis | Original material | Fraction | | | | |
|---|---|---|---|---|---|---|
| | | Fraction unextracted | $Fr_{60}$ | $Fr_{70}$ | $Fr_{alc.+et.}$ | $Fr_{80}$ |
| N* | 14.17 | 14.04 | 12.00 | 13.25 | 11.16 (11.88)‡ | 12.61 (13.96)‡ |
| Glucosamine | 4.59 | 4.02 | | 9.01 | | |
| Tolal non-amino sugars† | | | | | | |
| Indole r. | 7.51 | 6.30 | 9.56 | 14.15 | 8.77 | 9.34 |
| Orcinol r. | 7.22 | 5.81 | 9.09 | 13.65 | 8.26 | 8.80 |
| Diphenyl-amine r. I | 7.94 | 6.45 | | | | |
| Average | 7.56 | 6.19 | 9.33 | 13.90 | 8.52 (9.07)‡ | 9.07 (10.04)‡ |
| Acetyl | | 1.34 | | 2.82 | | |
| Ash | | 2.03 | 3.51 | 3.60 | 6.08 | 9.65 |

* Micro Kjeldahl. † Expressed as composed of equimolecular galactose and mannose.
‡ The figures in parenthcses show percentages on an ash-free basis.

TABLE IIb.

Analysis of the Fractions of Crude Serum Mucoid.

The figures express the content in equivalents per equivalent weight.

| Analysis | Original material | Fraction | |
|---|---|---|---|
| | | Fraction unextracted | $Fr_{70}$ |
| Glucosamine | 1.0 | 1.0 | 1.0 |
| Tolal non-amino sugars | 1.6 | 1.5 | 1.5 |
| Acetyl | | 1.4 | 1.3 |

Analysis of the Original Crude Product and its Fractions.

The results are embodied in Tables $II_a$–$II_b$.

The fractions as well as the original crude material gave the positive reaction with *p*-dimethylaminobenzaldehyde by the direct process of Osaki and Turumi and also that characteristic of acetylhexosamine by the indirect. The farction unextracted by boiling water was contaminated with phosphorus distinctly (ammonium molybdate test) and Liebermann-

Burchard test revealed the presence of sterols in it. In the remaining fractions sterols were absent but the last trace of phosphorus had not been removed.

We express a sincere gratitude to the Grant Committee for Scientific Researches of the Education Department for a grant towards the expenses of this work. H. Masamune.

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2) Suzuki, H., ibid., 1941, **34**, 167.
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6) Zanetti, Malys Jahresber. d. Tierchem., 1897, 31; 1903, 10.
7) Rimington, Bioch. J., 1940, **34**, 931.
8) Readers are referred particularly to Lloyd and Shore, Chemistry of the Proteins, 2nd Ed., J. & A. Churchill, London, 1938, pp. 425–452, where the present knowledge of denaturation and coagulation is beautifully summarized.
9) Hasegawa, Tohoku J. Exp. Med., 1943, **46**, 95. Oh-Uti, ibid., 1948, **49**, 193, 207. Asagi, ibid., 1949, **50**, 95.
10) Vid. especially Hamasato and Akakura, J. Bioch., 1941, **34**, 159, and Osaki and Yosizawa, Tohoku J. Exp. Med., in press.
11) Yasuoka and Sasaki, Tohoku J. Exp. Med., 1947, **49**, 5.
12) Bywaters, Bioch. Z., 1909, **15**, 322.
13) Rimington, Ergebn. Physiol., 1933, **35**, 712.
14) Hamasato and Akakura, J. Bioch., 1941, **34**, 159. Yasuoka, Tohoku J. Exp. Med., 1944, **46**, 261 (footnote ‡ by Masamune).
15) Osaki and Turumi, Tohoku J. Exp. Med., 1947, **49**, 11.
16) The authors tested the present preparation.
17) Hiyama, Tohoku J. Exp. Med., in press.

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# The Effect of Anoxia on the Instestinal Secretion.

By

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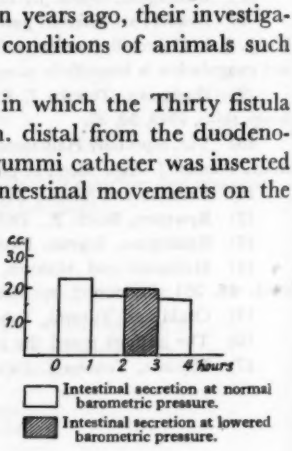
(Received for publication, July 26, 1949)

Although the effect of anoxia on the secretion of the enteric juice was studied by Northup and Van Liere[1] about ten years ago, their investigations were performed under non-physiological conditions of animals such as anesthesia and laparotomy.

In the present studies, the dog was used, in which the Thirty fistula was prepared with the small intestine 10–20 cm. distal from the duodenojejunal flexura. At the time of experiment a gummi catheter was inserted in the fistula, so as to avoid the influence of intestinal movements on the ejection of intestinal juice as possible.[2]

Placing the animal on the table in the large low pressure chamber, the rate of intestinal secretion for every one hour in non-feeding period was estimated. The barometric pressure in the chamber was lowered to the pressure corresponding to the altitude of 8 km. during one hour.




Comparing the output rate of enteric juice at normal, and lowered barometric pressure of the chamber, we could not find any definite difference among them, although some fluctuations of small magnitude were observed. The result suggests that the intestinal secretion in non-feeding period is not influenced by the anoxia of such severe degree as described above.

## Literature.

1 Northup and Van Liere, Proc. Soc. Exp. Biol. Med., 1939, **42**, 162.
2 Takahasi, Tohoku J. Exp. Med., 1949, **50**, 157.

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# Biochemical Studies on Carbohydrates.

## CVII. On Vitellomucoid.

### (A Correction of XVI Information.)

By

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(Received for publication, March 19, 1947)

Vitellomucoid of Onoe[1] was purified by electrodialysis and analyzed by our recent methods. Acetyl determination gave a value corresponding to two equivalents per equivalent of hexosamine. This accords with the finding, as Osaki and Turumi[2] already observed, that this mucoid develops with Ehrlich reagent red coloration with the one-band spectrum but not the one with the two-band spectrum, because the former reaction is due, according to Hiyama[3], to a structure wherefrom two molecules of a volatile monocarboxylic acid are produced by hot acid. The molecular ratio of mannose to glucosamine has been established to be 11 : 10.

## Experimental.

### Preparation and Properties of Vitellomucoid.

The mucoid was prepared and purified according to the second method of Onoe. Since the preparation contained about 14% ash, it was subjected to electrodialysis (Pauli apparatus, 200 volts). 500 mgm. were adapted for the purpose as 1% solution. It took 8 days, until the water in the outer compartments, which was changed from time to time, showed no pH shift. The occurring precipitate was centrifuged off and the clear supernatant liquid distilled under reduced pressure to about 10 cc. Then 150 cc. of absolute alcohol and 20 cc. of ether were added and the deposit was washed twice with absolute alcohol and dried in a desiccator over $H_2SO_4$. A transparently soluble product weighing 89 mgm. was obtained.

It gave by the direct process of Osaki and Turumi the positive colour reaction with *p*-dimethylaminobenzaldehyde, but by the indirect no dye characteristic of acetylhexosamine. Phosphorus was detected but did not reach beyond a trace. The quantitative-analytical figures and optical

rotatory power are listed in Table I.

TABLE I.

Analysis and Optical Rotation of Pure Vitellomucoid.

| Analysis | In per cent | In equivalents per equivalent weight |
|---|---|---|
| N | 11.5 | |
| Mannose | | |
| Indole r. | 24.0 | |
| Orcinol r. | 23.62 | |
| Diphenylamine r. I | 23.57 | |
| Mean | 23.73 | 1.1 |
| Glucosamine | 21.5 | 1.0 |
| Hydrolysable volatile acid as acetyl | 10.31 | 2.0 |
| Ash | 1.10 | |
| $[\alpha]_D^{19.5}$(in $H_2O$) | $\frac{-0.758\times 100}{1\times 2.478}=-30.59°$ | |

The costs of this work were covered by a grant from the Grant Committee for Scientific Researches of the Education Department, which is gratefully acknowledged.

## References.

1) Onoe, J. Bioch., 1936, **24**, 1.
2) Osaki and Turumi, Tohoku J. Exp. Med., 1947, **49**, 11.
3) Hiyama, Tohoku J. Exp. Med., in press.

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# Biochemical Studies on Carbohydrates.

## CVIII. Amino Sugar in the Glycidamin Making up Chondromucoid.

By

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(Received for publication, April 2, 1947)

Sasaki[1] reported that tendomucoid $\beta$ contains both glucosamine (in the glycidamin-moiety) and chondrosamine (in the chondroitinsulfuric acid-moiety) and Osaki[2] expressed a similar opinion on sclera mucoids. Viewed from these recent findings, Hisamura's announcement[3] that the glycidamins in chondro- and osseo-mucoid contain chondrosamine as their hexosamine constituent looks to require reinvestigation, because cartilage and bone are mesenchymal derivatives similar to tendon and sclera. So I recrystallized Hisamura's preparations left over of hexosamine benzoate to see if their melting point rises thereby to approach that of glucosamine benzoate. The results came out as expected. Regretfully, however, the substance was too little for a detailed study which compelled me to start with separation of the glycidamins. The present account deals with the work on the hexosamine in chondromucoid-glycidamin. This glycidamin was prepared by removing chondroitinsulfuric acid from the mucoid with glycol and the hexosamine was isolated by fractioning its hydrolysate with butanol as from the jelly-forming proteins of toad and frog eggs.[4]

The obtained hydrochloride showed properties, chemical and physical, of glucosamine hydrochloride and gave an $\alpha$-hydroxynaphthylidene derivative identical with the corresponding derivative of this hexosamine.

### Experimental.

### Chondromucoid and its Glycidamin.

Chondromucoid was prepared from whale nasal cartilage. The extraction and following precipitation were performed according to the first method of C. Th. Mörner[5] and the precipitates washed with a dilute HCl

of pH 1.8 exhaustivily and then with alcohol and ether in sequence and dried.[6]

To separate the glycidamin moiety, 1 gm. of the above mucoid was kneaded with a small portion of glycol and after increasing glycol to 20 cc., 1 n. NaOH added in drops to slightly alkaline, whereby the mixture took pasty appearance, the mucoid having been dissolved almost entirely. The glycidamin was precipitated by bringing the pH to 6.5 with several drops of 1 n. HCl and separated by prolonged centrifugation. The centrifugate was washed twice with 20 cc. glycol with repeated centrifugations and many times with alcohol. Alcohol was replaced with ether, which was evaporated off. Yield, 515 mgm. It gave positive biuret, Molisch, Osaki-Turumi (both the direct and indirect) and Hiyama[7] reactions. The naphthoresorcinol test for hexuronic acid (Neuberg-Saneyoshi) resulted in negative. Quantitative analysis gave: nitrogen 12.82%, hexosamine 12.63%, galactose 12.05% (the indole method: 12.2%, the orcinol method: 11.5%, the *α*-naphthol method I: 12.4%), hydrolysable sulfur 1.34%, ash 0.43%.

### Isolation of the Hexosamine from the Hydrolysate of the Glycidamin.

The procedure described in the third report concerning the jelly of toad and frog eggs[4] was followed with minor modifications as below.

1.8 gm. of the glycidamin separated was hydrolyzed with 20 cc. of 20% HCl containing 0.8 gm. of stannous chloride by boiling under reflux for 15 hours on a sand bath (The substance was dissolved in advance on a water bath). Without removing the dark sediments, which had appeared during the heating, the hydrolysate was diluted with double the volume of water and bubbled with $H_2S$-gas. The excess $H_2S$ was expelled by aeration and the precipitates and the above sediments were filtered off together and washed with hot water on the funnel and followingly in a beaker. The yellowish transparent filtrate combined with the washings was evaporated under reduced pressure (40°C) to a syrup, evaporated again twice with 5 cc. of water and finally taken up in 14 cc. of water for fractionation with butanol in a Dakin's apparatus. Extraction was continued for 6 hours and the inspissated fluid (1.5 cc.) was diluted with water, the appearing precipitates centrifuged off and the supernatant liquid condensed by vacuum distillation to about 0.5 cc. To this condensate 0.5 cc. of concentrated HCl and 5 cc. of absolute alcohol were added and some precipitates filtered off. And to the filtrate was added acetone cautiously until faint cloudiness occurred. Crystallization began after a while. Acetone was supplemented thereafter from time to time and when oily deposit began to appear, the crystals were separated, washed with absolute alcohol and ether and dried in a desiccator ($CaCl_2$). Yield, 71 mgm.

The crude product was dissolved in 1 cc. of water mixed with a few drops of concentrated HCl. On addition of 10 cc. of absolute alcohol beautiful crystals came out in plates (hexagonal, tetragonal and trigonal). After further addition of a little acetone and keeping the mixture in an ice chest overnight, the crystals were collected. Yield, 60 mgm.

The substance began browning at about 180°C, but did not melt even at 250°C. Red colouration developed by *p*-dimethylaminobenzaldehyde, when it had been heated preliminarily with an alkaline acetylacetone solution. The positive chloride test was given. It reduced hot Fehling solution.

N (micro Kjeldahl method).

2.85 mgm. substance, 1.349 cc. 0.01 n. acid neutral. by $NH_3$.

$C_6H_{13}O_5N \cdot HCl$. Calc. 6.50%, found 6.63%.

Optical rotation (in water).

5 minutes after dissolution:

$$[\alpha]_D^{18} = \frac{1.389 \times 100}{1.425 \times 1} = +97.5°,$$

24 hours after dissolution:

$$[\alpha]_D^{18} = \frac{1.035 \times 100}{1.425 \times 1} = +72.6°.$$

The mutarotation proceeded as is seen in Table I.

TABLE I.

Course of Mutarotation in Water of the Hexosamine hydrochloride from Chondromucoid-glycidamin.

| Time after dissolution min. | $\alpha$ deg. | $[\alpha]_D^{18} = \frac{\alpha \times 100}{1.425 \times 1}$ deg. |
|---|---|---|
| 5 | +1.389 | +97.5 |
| 15 | +1.339 | +94.0 |
| 30 | +1.270 | +89.1 |
| 60 | +1.194 | +83.8 |
| 60×2 | +1.115 | +78.2 |
| 60×4 | +1.039 | +72.9 |
| 60×24 | +1.035 | +72.6 |

The initial $[\alpha]_D$ extrapolated is +100°. This and the rotation at equilibrium agree perfectly with Irvine and Earl's values[8] of *α*-glucosamine hydrochloride.

The 2-Hydroxynaphthylidene Derivative of the Hexosamine in Question.

40 mgm. of the hexosamine hydrochloride above were condensed with

2-hydroxynaphthaldehyde by the method of Jolles and Morgan[9]. The excess of the aldehyde was removed by means of ether. The crude product (48 mgm.) melted at 187–190°C (decomp.) and the twice recrystallized from hot methanol (38 mgm.) at 202°C (decomp.). Most of the crystals took a needle form and the remaining the form of plate. Its mixture with the corresponding Schiff's base of glucosamine (below) with F. P. of 200°C (decomp.) melted without any depression.

N (micro Kjeldahl method with antecedent reduction by means of HI and red phosphorus).

6.50 mgm. substance, 1.881 cc. 0.01 n. acid neutral. by $NH_3$.

$C_{17}H_{19}O_6N$. Calc. 4.20%, found 4.13%.

Optical rotation (in methanol). It had the initial rotation (12 minutes after dissolution) $[\alpha]_D^{16}=\frac{0.695\times 100}{0.343\times 1}=+202.6°$ and the equilibrium rotation $[\alpha]_D^{18}=\frac{0.601\times 100}{0.343\times 1}=+175.2°$ (measured 24 hours after dissolution), which are in accord with those of the corresponding derivative of glucosamine hydrochloride (s. below). The equilibrium was secured in 6 hours.

A note on 2-hydroxynaphthylidene-glucosamine.

According to Jolles and Morgan this Schiff's base of glucosamine crystallizes in tufts of thin needles. The authors only described to have used "glucosamine hydrochloride," and so it was most probably a usual preparation, namely the *α*-isomer. Although I subjected *α*-glucosamine hydrochloride (Ledderhose) to the condensation by strictly following their technique (The excess aldehyde was extracted off with chloroform and ether), the product after one recrystallization from hot methanol took half and half the forms of tetragonal thick plate and of thin needle. By the second recrystallization it changed to a great part into needles, but needles alone could not be obtained by further recrystallization and the melting point remained constant. 130 mgm. were yielded from 150 mgm. of *α*-glucosamine hydrochloride, and as to the melting point (200°C (decomp.)), solubility and nitrogen content (4.17%)[10] the substance coincided with Jolles and Morgan's. And hydrolysis according to the same authors gave a nearly theoretical amount of glucosamine hydrochloride[11] (13.5 mgm. glucosamine hydrochloride were given from 25 mgm. of the base). I could not measure its rotation of light of wave length 5461 Å as the authors, but the initial $[\alpha]_D$ was $+203.7°$ ($[\alpha]_D^{22}=\frac{0.780\times 100}{0.383\times 1}=+203.7°$) after 6 minutes from dissolution, which changed to $+174.4°$ ($[\alpha]_D^{22}=\frac{0.668\times 100}{0.383\times 1}=+174.4°$) in 24 hours.

I have already proved that the corresponding Schiff's bases of $\alpha$- and of $\beta$-chondrosamine and their mixture all melt at one and the same temperature[12]. Therefrom it is analogized that my product above was a mixture of the bases of $\alpha$- and $\beta$-glucosamine and largely composed of the former.

The costs of this work were defrayed by a grant from the Education Department through the Grant Committee for Scientific Researches.

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11) The product had the crystalline form of plate and did not melt even at 280°C on heating. The initial rotation (in water) was $[\alpha]_D^3 = +95.5°$ which changed to $+73.3°$ in 24 hours. It gave the strongly positive Elson and Morgan test for hexosamine.
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# A New Device to Get a Radiological Section Figure of Body.

Preliminary Report.

By

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(Received for publication, July 27, 1949)

To get a radiological section figure of body, the writer worked on a new method of radiography, " the rotatography," which was first projected and studied by Y. Koga. The name rotatography was given because the x-ray exposure is made from various directions, rotating the tube around the object. A section figure of body is obtained by analysis of the rotatographs. To get a whole section figure of body, one must rotate the tube 180 degrees at least.

If radiographies are made every 15 degrees or so of rotating angles on different successive parts of a film, so we get a set of " the discontinuous rotatographs." These rotatographs are essentially usual radiographs, but of different projection. The analysis of the rotatographs begins with a careful rearrengement of the geometric conditions of rotatography. Tracing back the conditions of projection along the series of rotatographs will furnish the aimed section figure.

If the exposure is made continuously through a slit during the whole course of rotatography while the film slides, we get a peculiar graphical sight of radiograph on a film. A whole section of a part of body is thus expressed as a kind of curves. The analysis of this " continuous rotatographs " is rather difficult. Over twenty laws were found out for it.

The details will be published in a later issue of this Journal.

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# Experimental Study of Intraaxillary Vaccination of B. C. G.

By

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(Received for publication, April, 19, 1947)

Yoshio Takahashi,[1] in 1943, made public intraaxillary vaccination of B.C.G. Iwao Kanno[2], afterwards, suggested intraaxillary inoculation as a method of vaccination of B.C.G. more rational from the clinical point of view than the other methods. He reported the school boys and guinea-pigs did not suffer injury and the local lesion through the vaccination performed in this manner, and reacted positively to tuberculin more rapidly and more strongly than when the intracutaneous method was employed. He had 115 school boys who were vaccinated with 0.06 mg. of B.C.G. intraaxillary and 86 (74.8 per cent) had become tuberculin positive 22 days after the vaccination. In 111 there were no local reactions, and in 4 (3.5 per cent) the regional lymph glands were swollen. He saw 110, 45 days after the vaccination, and found that 110 (100 per cent) had become tuberculin positive. In 104 there were no local reactions, and in 6 (5–4%) the regional lymph glands were swollen. He saw 110, 102 days after the vaccination, and found that 110 (100 per cent) had become tuberculin positive. In 108 there were no local reaction, and in 2 (1.8%) the regional lymph glands were swollen. If we want to do the intraaxillary vaccination, we must order the one who is to be vaccinated to place his hand on his occiput. By this behaviour, a hollow will be formed in the axillary region, into the bottom of which we prick a needle of a syring deeply back-upwards about five centimenters, and inject B.C.G. 0.1 mg. in 1 cc.

I wanted to resolve the questions of why the local lesion did not appear by this method. Let us think about why the local lesion will appear. Abscessformation is, first, more likely to occur in a naturally infected person (Koch's phenomenon). Second, a great individual variation in susceptibility and individual difference in the resistance to B.C.G. virus should

be considered. Third, the homogeneity, the density, the quantity of the emulsion and the place in which B.C.G. is injected should be considered. According to Rosenthal's[3] microscopical studies, the cutaneous and subcutaneous lymphatics soon after inoculation was of great significance. Following the multiple puncture method, the lymphatics contained a few polymorphonuclear leucocytes which had phagocytosed tubercle bacilli. On the other hand, following a large single intracutaneous dose, the lymphatics were widely dilated and stuffed with cells and bacteria- amounting to a partial obstruction.

## Experiment.

I would, therefore, examine by which way and how rapidly B.C.G. would be absorbed, and where it would stay. It was determined, preparatorily, how many mg. of B.C.G. in the blood and the lymph could be isolated. This was tried according to the method of cultivation which was reported by Takashi Katakura, Sutemi Oka, Tamura and Ishikawa[4] and known as the sulphric acid pepsin method in Japan. The emulsion of B.C.G. was put into 3 cc of blood mixed with 18 cc of 0.1% saponin solution, containing calcium chloride at the ratio of 0.25%, and left to stand for three hours. Then it was centrifuged at a ratio of three thousand revolutions per a minute for thirty minutes, and the supernatant fluid was thrown away. Distilled water was poured into the sediment. This was centrifuged once more in the same manner, after being washed. The supernatant fluid was thrown away and a few drops of 4% sulphric acid solution containing pepsin was dropped into the sediment, and it was left to stand for thirty minutes and then inoculated into the Oka-Katakura medium, which was reported by Sutemi Oka and Takashi Katakura.[5]

I succeeded in isolating 1/10.000,000 mg. of B.C.G. in 3 cc of the blood (Table I).

Table I.

Results of bloodculture (preparatory).

| No. \ mg. of B.C.G. | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
|---|---|---|---|---|
| 1 | ## | 2 colonies | 1 colony | 1 colony |
| 2 | ## | 4 colonies | 1 colony | 0 |
| 3 | ## | 3 colonies | 2 colony | 0 |
| 4 | ## | 4 colonies | 2 colony | 1 colony |
| 5 | ## | 3 colonies | 2 colony | 0 |

Then dogs were used for study. Five mg. to 28 mg. of B.C.G. were injected into the axillary region of dogs, which weighed 6 kg. to 20 kg. Their lymph, which was obtained from ductus thoracicus, and the blood, which was obtained from V. jugularis, were cultivated according to the sulphric acid pepsin method, after the inoculation of B.C.G. A sterilized canula was directly inserted into ductus thoracicus near V. jugularis to obtain lymph. The lymph was centrifuged at a ratio of 3,000 revolutions a minute, and the supernatant fluid was thrown away. A few drops of sulphric acid solution containing pepsin was added to the sediment and left to stand for thirty minutes and inoculated into Oka-Katakura medium. These were placed into a incubator kept at 38.0°C for two months. The blood, obtained sterilely from V. jugularis, was inoculated into Oka-Katakura medium according to the sulphric acid pepsin method and placed in the incubator kept at 37.0°C for two months. As Table II shows, it was not possible to isolate B.C.G. from the lymph after the intraaxillary vaccination, but from the blood, it was possible to isolate 18 minutes to 32 hours after injection. The lymph vessels of the axillary region show a

TABLE II.

Results of blood-, lymphculture after intraaxillary vaccination.

| No. of anim. | 1 | | 2 | | 3 | | 4 | 5 |
|---|---|---|---|---|---|---|---|---|
| Weight (Kg) | 9 | | 9 | | 7 | | 12 | 14 |
| Quant. of B.C.G. (mgr.) | 5 | | 18 | | 28 | | 28 | 28 |
| | Lymph | Blood | Lymph | Blood | Lymph | Blood | Blood | Blood |
| Before Vacc. | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) |
| 11′ | | | | | | | | |
| 15′ | | | | | | | | |
| 18′ | | | | | | | (卌) | 82col. |
| 25′ | | | | | | (卌) | (卌) | (卌) |
| 30′ | (−) | (−) | (−) | 60col. | (−) | | | |
| 1 h | (−) | 2col. | (−) | 50col. | (−) | 50col. | | |
| 2 h | (−) | (−) | (−) | 6col. | (−) | 11col. | (卌) | 35col. |
| 3 h | (−) | (−) | (−) | 10col. | (−) | 23col. | | |
| 4 h | | | | | (−) | 8col. | | |
| 7 h | | | | | (−) | 8col. | | |
| 9 h | | | | | (−) | 5col. | | |
| 11 h | | | | | (−) | 18col. | | |
| 16 h | | | | | (−) | 3col. | | |
| 19 h | | | | | (−) | 15col. | | |
| 32 h | | | | | (−) | (——) | | |

variation[6)7)], so they sometimes flow into ductus thoracicus and sometimes into V. subclavia and V. jugularis. It, therefore, does not mean that B.C.G., which was injected into the axillary region, was not absorbed by the lymph vessels (and unable to be isolated in the lymph, which is obtained from ductus thoracious). Wallgren[8)] has seen, after the intracutaneous vaccination, an enlargement of the regional lymph glands, followed by caseous degeneration, suppuration, and fistula formation. It is obvious that B.C.G. is spread by way of the lymphatics, from the primary focus to the regional lymph glands. Following the multiple puncture method, Rosenthal[3)] has reported that the lymphatics contained a few polymorphonuclear leucocytes, which had phagocytosed tubercle bacilli. On the other hand, following a large single intracutaneous dose, the lymphatics were widely dilated and stuffed with cells and bacteria. Therefore, in spite of the fact that B.C.G. is not isolated from the lymph, it should spread by way of the lymphatics. S. Katsura[9)] has reported that paratyphoid B bacilli would be absorbed directly by blood vessels. And according to my experiments, in the shotness of time and the number of B.C.G. isolated, B.C.G. must be absorbed directly by blood vessels as well as by lymphatics.

Neither was B.C.G. isolated in the blood which was obtained from V. jugularis after subcutaneous injection near the axillar.

B.C.G. was not isolated in the blood, which was obtained from V. jugularis, either after intracutaneous vaccination of the same dose the intraaxillary and subcutaneous vaccination near the axillar.

These experiments seem to show that B.C.G. would be absorbed more easily and more rapidly from intraaxillary than from subcutaneous or intracutaneous injection.

Next, rats were slaughtered after intraaxillary, subcutaneous, and intracutaneous vaccination of 15 mg. of B.C.G., and the viscera was crushed in mortars, and some 4% sulphric acid solution was poured into them. They were centrifuged at a ratio of 3,000 revolutions a minute after being left to stand for 30 minutes, The supernatant fluid was thrown away, and the sediment was inoculated into Oka-Katakura medium. They were placed in a incubator kept at 37.0°C for two months. In the case of the intraaxillary vaccination, B.C.G. could be isolated from the lung, heart, spleen and kindney, but not from the liver, 10 minutes after injection (Table III).

In the case of the subcutaneous or intracutaneous vaccination, B.C.G. could not be isolated from the lung, heart, spleen, kidney or liver 10 minutes to 3 hours and 30 minutes after injection of the same does as the former.

In the case of the intraaxillary vaccination, there may be multiple foci of quantities of bacilli, which call forth tissue responses that are accompanied by good drainage by the way of patent lymph channels. Following

TABLE III.

Results of visceraculture after intraaxillary vaccination.

| No. of animal | Quant. of B.C.G. | Time of Kill | Lung | Heart | Spleen | Kidney | Liver |
|---|---|---|---|---|---|---|---|
| 1 | 15mg. | 10′ | 1col. | 1col. | 2col. | 2col. | (−) |
| 2 | 15mg. | 10′ | 1col. | 1col. | 2col. | 2col. | (−) |
| 3 | 15mg. | 10′ | 1col. | 1col. | 1col. | 1col. | (−) |
| 4 | 15mg. | 15′ | 1col. | 1col. | 1col. | 1col. | (−) |

the subcutaneous or intraneous vaccination of the same amount of vaccine, there may be only one local lesion which, because of the massive concentrated does of bacilli, cause local tissue destruction that is further enhanced by the partial blockade of the lymph channels.

Next, I observed the mode of the absorption of a mono-sodium salt solution of phenolsulphthalein (Sankyo) after intraaxillary, intracutaneous, and subcutaneous injection of 0.6% solution, which applied to man. This dye is absorbed by way of the blood vessels, after injection into the serous cavity, and excreted very well through kidney.[10] The urine excreted was collected, and the "phthalein" excreted was estimated colorimetrically after injection of one measured cubic centimeter of the solution. The quantity excreted was estimated, using the Dunning colorimeter. One tenth of the urine excreted was rendered alkaline by the addition of a little solution of sodium hydroxide, and the alkaline urine was diluted with water to make it measure one hundred cubic centimeters. The open ampule, containing the diluted alkaline urine, was then placed in the center hole of the colorimeter box and compared with the number test ampules until the one that most nearly matched the specimen in color was found.

The quantity of this excreted dye was estimated 3 to 4 hours after being injected. This dye was absorbed intraaxillary first, intracutaneously second, and subcutaneously third.

Five cubic centimeters of a 2% solution of methylene blue (Grübler) was observed after intraaxillary and subcutaneous injection of man. The standard solution was made by me. A 0.0001% solution of this dye was found in the urine. If the excreted contains methylene blue, it appears blue. It seems to be absorbed by way of the blood vessels and excreted in the urine after injection.[10]

This dye was estimated from the urine which was excreted 3 hours after the injection. According to my experiments, methylene blue, when injected intraaxillarily, was found in the urine, but, when injected subcutaneously, it could not be found (Table IV).

Next, 10 cc of a 2% solution of Congo-red (Grübler) was observed after intraaxillary and subcutaneous injection of a dog. The standard

TABLE IV.

The mode of the absorption of methylene blue.

| Number of Experiment | I | | II | | III | |
|---|---|---|---|---|---|---|
| Mode of Injection / Time | Subcut | Intraax. | Subcut | Intraax. | Subcut | Intraax. |
| 30′ | (−) | 0.0378% | (−) | (−) | (−) | 0.0154% |
| 1 h | (−) | 0.0345% | (−) | 0.0093% | (−) | 0.0082% |
| 2 h | (−) | 0.0555% | (−) | 0.0074% | (−) | 0.0060% |
| 3 h | (−) | 0.0350% | (−) | 0.0060% | (−) | 0.0055% |
| Total | (−) | 0.1628% | (−) | 0.0227% | (−) | 0.0351% |

solution was made by me also. A 0.0001% solution of this dye was found in the blood. If the blood contains this dye, the serum appears red. This can be confirmed by adding a few drops of hydrochlorid acid. If the serum contains this dye, it become blue. This colloid dye seems to be absorbed by way of the lymphatics only and passes into the blood vessels. If it is injected venously, it stays in the blood longer, and it is not excreted in the urine. Five cubic centimeters of blood was procured, and its serum was observed to estimate the amount of congored. This dye, when injected intraaxillarily, was absorbed more than injected subcutaneously (Table V).

TABLE V.

The mode of the absorption of congored.

| Time | Subcut | Intraax |
|---|---|---|
| 12′ | | 0.0015% |
| 25′ | | 0.0025% |
| 35′ | 0.0007% | 0.0020% |
| 40′ | 0.0005% | |
| 1 h | 0.0003% | 0.0020% |
| 2 h | (−) | |
| 3 h | (−) | 0.0020% |

Last, 2 cc of a 2% solution of indigocarmin was observed after intraaxillary and subcutaneous injection of man. The standard solution was made by me also. A 0.0001% solution of this dye was found in the urine. This dye, when injected, is excreted in the urine. If the urine contains this dye, it appears blue. The molecule of this dye is a cross between a crystalloid and a colloid and seems to be absorbed both by way of the lymphatics and blood capillaries. The urine, which was excreted 3 hours and 30 minutes after the injection, was examined to estimate this dye. This dye, when injected into intraaxillary region, was found in the urine, but, when injected subcutaneously, it could not be found (Table VI).

TABLE VI.

The mode of the absorption of indigocarmin.

| Number of Experiment | I | | II | |
|---|---|---|---|---|
| Mode of Injection / Time | Subcut | Intraax | Subcut | Intraax |
| 30′ | (−) | (−) | (−) | (−) |
| 1 h | | | | 0.066% |
| 1h30′ | (−) | 0.280% | (−) | |
| 2 h | | | | (±) |
| 2h30′ | (−) | 0.175% | (−) | |
| 3 h | | | | (−) |
| 3h30′ | (−) | (−) | (−) | |

## DISCUSSION.

I must first answer the question of how B.C.G., inoculated intraaxillarily will be absorbed. According to my experiments, B.C.G. could be shown in the blood obtained from V. jugularis 18 minutes after injection. It seems to be absorbed directly by blood capillaries as well as by the lymphatic capillaries.

I must, secondly, answer the question of how rapidly B.C.G., inoculated intraaxillarily, will be absorbed. B.C.G. was able to be isolated in the heart, lung, kidney, and spleen of rats 10 minutes after the intraaxillary vaccination.

I must, thirdly, answer the question of where B.C.G. will stay. According to may experiments, it stays in the lung, kidney and spleen. It show according to my experiments that B.C.G. and other dyes, when inoculated into the intraaxillar region, were and more rapidly absorbed than when they were inoculated subcutaneously and intracutaneously. We can easily suppose that the intraaxillary region has more well-developed lymphvessels than subcutaneous and intracutaneous sites. In the intraaxillary region there is a large hole which has a spongy construction, so that when substance is injected into the intraaxillary region, it spreads throughout the entire hole and is absorbed easily. Therefore, there is no doubt that B.C.G. vaccinated intraaxillarily, and absorbed by the blood capillaries and lymph capillaries, is absorbed so easily and readily, that the local lesion does not appear.

## SUMMARY.

I can resolve the question of why the local lesion does not occur when B.C.G. is vaccinated intraaxillarily. The reasons are the following:

1. B.C.G., which is inoculated into the intraaxillary region, seems to be absorbed by way of the blood capillaries as well as lymphatic capillaries.

2. In the intraaxillary region there is a large hole which has a spongy construction and well-developed lymph vessels and blood vessels.

3. The substances (bacteria, dyes) which are inoculated into the intraaxillary region, are absorbed so readily, rapidly, and easily that the local lesion does not occur.

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Fig. 1. The mode of the absorption of phenolsulphthalein.

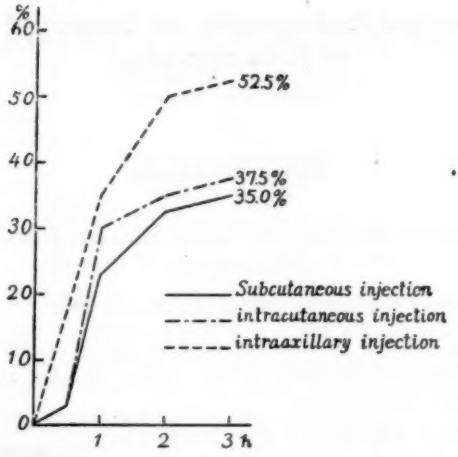
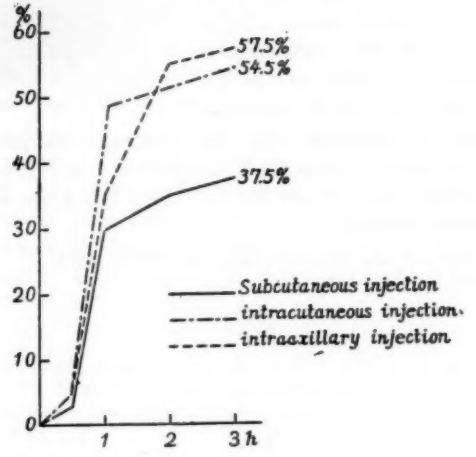



Fig. 2. The mode of the absorption of phenolsulphthalein.



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# Cross-section Radiography, an Improved Method of Rotatography.*

Preliminary Report.

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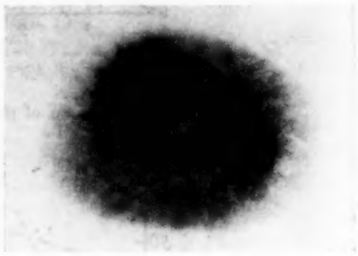
(Received for publication, July 27, 1949)

After careful studies of rotatography to get a radiological picture of transsection of body, there arose to the author an idea of a new method, "the cross-section radiography."



A cross-section radiograph can be taken by integrating the discontinuous rotatographs. Instead of a simple lineal sliding of film in ordinary rotatography, the film slides here in each exposure, and rotates in the rotary cassett-holder as many angles as the tube-cassett-pendulum when the latter moves for the next exposure. A whole cross-section radiograph is obtained after the repeated integrating exposures. The technique is indeed somewhat complicated but the resulting cross-section radiograph is quite intelligible. The figure shows a cross-section radiograph of the forearm.

A full technique will be published in this Journal.

* Read at the Annual Meeting of the Japanese Radiological Society, May 2, 1949, Sendai.

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# A Contribution to the Stadie-Wu's Method of Hemoglobinometry.

By

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The Stadie-Wu's method of hemoglobinometry[1)2)3)] has been generally appreciated because of its accuracy and simplicity in procedure. I noticed, however, that this method has an inconvenience that the colorimetric mixture is not stable in the color-tone, the latter changes fairly rapidly in course of time after it is prepared by adding ferricyanide and cyanide solution to the sample. The following report is the result of the experiments performed to eliminate such difficulty found in the original procedure.

## Experimental.

### Experiment I.

The colorimetric mixture of human blood prepared on the first day by Stadie-Wu's method was compared every following day with the mixture freshly prepared each day of the blood of the same person. A Duboscq type colorimeter was used. As the amount of hemoglobin in blood changes from day to day even in the same individual, the actual concentration of blood hemoglobin was estimated everyday by Van Slyke's gasometric method. Four sets of such experiments were performed and the results obtained are as shown in Table I and Fig. 1.

From these results it is evident that no parallelism exists between the

Table I.

Comparison of oxygen capacity with colorimetric reading by the original Stadie-Wu's cyanhemoglobin method.

| Experimental Day | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Hemoglobin Content Vol. % $O_2$ Capacity | 18.1 | 18.2 | 18.1 | 18.1 | 18.3 |
| Colorimetric Reading* | 8.0 | 9.3 | 9.4 | 9.4 | 9.5 |

| Experimental Day | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Hemoglobin Content Vol. % $O_2$ Capacity | 18.0 | 18.3 | 18.3 | 18.0 | 18.0 |
| Colorimetric Reading* | 8.0 | 9.3 | 9.4 | 9.2 | 9.2 |

| Experimental Day | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Hemoglobin Content Vol. % $O_2$ Capacity | 19.4 | 19.4 | 19.2 | 19.0 | 19.0 |
| Colorimetric Reading* | 8.0 | 9.1 | 9.5 | 9.2 | 9.5 |

| Experimental Day | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Hemoglobin Content Vol. % $O_2$ Capacity | 19.4 | 19.2 | 19.2 | 19.3 | 19.6 |
| Colorimetric Reading* | 8.0 | 9.3 | 9.4 | 9.2 | 8.6 |

* The colorimeter cup of the first day's sample was set at 8.0 for the sake of convenience.

true hemoglobin content (oxygen capacity) and the colorimetric readings, the fairly marked change in colorimetric reading occuring during the period from the first to the second day.

## Experiment II.

This time the mixture prepared on the first day was compared every following day with the freshly prepared but by a slightly modified method, the details of which is as follows:

| Steps of procedure | Original method | Modified method with heating |
|---|---|---|
| 1 | 4% ammonia | 4% ammonia |
| 2 | 0.2% potassium ferricyanide | 0.2% potassium ferricyanide |
| 3 | Let it stand for 20 minutes at room temperature | Heat the solution to 50~60°C for 20 minutes |
| 4 | 0.1% KCN | 0.1% KCN |
| 5 | Colorimetry after 5 minutes at room temperature | Colorimetry after 5 minutes at room temperature |

The colorimetric readings and gasometric hemoglobin content were compared as in Experiment I. The results of four such experiments are shown in Table II and Fig. 2.

Contrary to the results of Experiment I, there is a satisfactory relation between two sets of values, i.e. oxygen capacity and colorimetric readings of the modified method.

*Fig. 1*

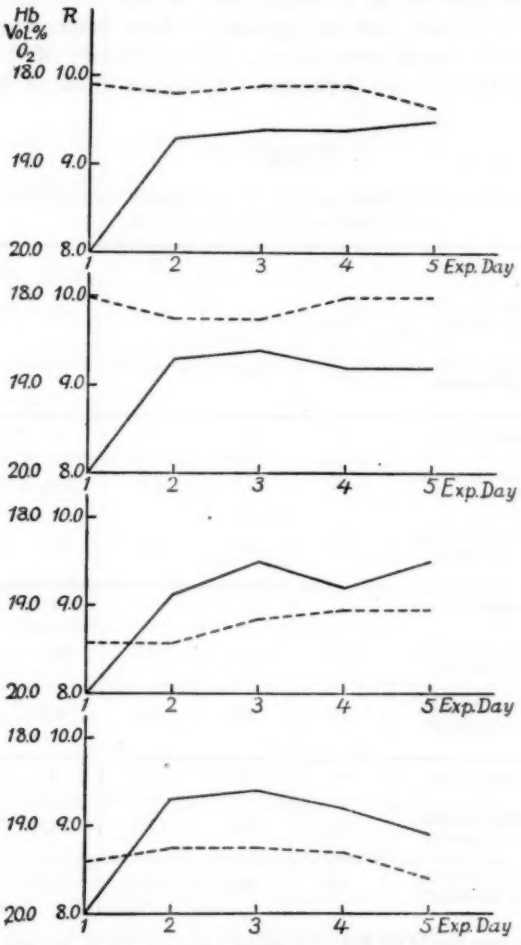


Fig. 1. Absence of realtionship between hemoglobin concentration and colorimetric reading (R) by Stadie-Wu's method.

———: Colorimetric reading.

- - - - - -: Oxygen capacity by gasometric measurement.

## Discussion.

Needless to say, the mixture for colorimetry has to be stable in its color-tone. On this point the Stadie-Wu's method in its original form has a demerit for all its other merits. Concerning to it, K. Matsuda[4] inferred that the instability of the color-tone of the solution prepared by the original Stadie-Wu's method would be dependent on the incompleteness of the reaction of ferricyanide and hemoglobin to form methemoglobin in 20 minutes at ordinary room temperature. As the velocity of this reaction is increased when the reaction (hydrogen ion concentration) of the medium is

## Table II.

Comparison of oxygen capacity with colorimetric reading by the modified cyanhemoglobin method.

| Experimental Day | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Hemoglobin Content Vol. % $O_2$ Capacity | 18.1 | 18.2 | 18.1 | 18.1 | 18.3 |
| Colorimetric Reading Method of Heating* | 8.0 | 8.1 | 8.2 | 8.1 | 8.3 |
| Experimental Day | 1 | 2 | 3 | 4 | 5 |
| Hemoglobin Content Vol. % $O_2$ Capacity | 18.0 | 18.3 | 18.3 | 18.0 | 18.0 |
| Colorimetric Reading Method of Heating* | 8.0 | 8.2 | 8.5 | 8.5 | 8.3 |
| Experimental Day | 1 | 2 | 3 | 4 | 5 |
| Hemoglobin Content Vol. % $O_2$ Capacity | 19.4 | 19.4 | 19.2 | 19.0 | 19.0 |
| Colorimetric Reading Method of Heating* | 8.0 | 8.1 | 8.4 | 8.6 | 8.6 |
| Experimental Day | 1 | 2 | 3 | 4 | 5 |
| Hemoglobin Content Vol. % $O_2$ Capacity | 19.4 | 19.2 | 19.2 | 19.3 | 19.6 |
| Colorimetric Reading Method of Heating* | 8.0 | 8.5 | 8.5 | 8.5 | 8.1 |

* The colorimeter cup of the first day's sample was set at 8.0 for the sake of convenience.

*Fig. 2*

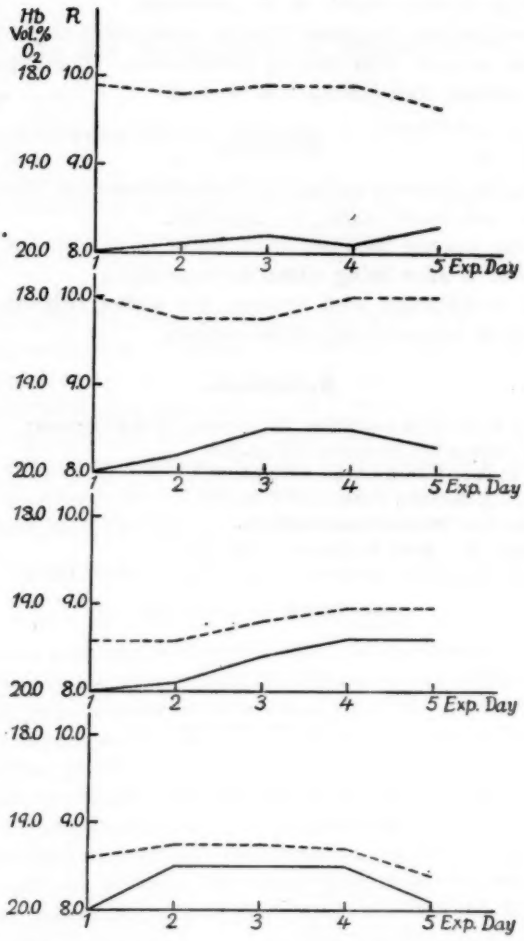


Fig. 2. Parallel relationship between hemoglobin concentration and colorimetric reading (R) by the modified method.

———: Colorimetric reading.

- - - - -: Oxygen capacity by gasometric measurement.

brought to the neutral or rather to the acid range (pH=6~7)[5][6] he tried as a modified technique in the preparation of the colorimetric solution to hemolyse the blood in distilled water (not in ammonia) and to add ferricy-

anide solution to it. Then after 30 minutes at room temperature ammonia and potassium cyanide were added. By this modification he said he was able to make the mixture stable in its color-tone.

The present author considered that the same effect might be produced by heating the solution after adding ferricyanide. The experiments reported above verified such inference to be true.

SUMMARY.

1) In the Stadie-Wu's method for hemoglobinometry the colorimetric mixture is not sufficiently stable in color-tone.

2) But the mixture becomes much stable in its color-tone when it is heated at 50~60°C after being added ferricyanide.

3) This modification with heating, the author believes, can be recommended as an improvement of the method.

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# On the Persistence of Brain-Waves.

By

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It is desirable for scientific studies of brain-waves to express them quantitatively with some measures. Motokawa[1] has proposed to use three statistical measures, i.e. mean amplitude $\bar{A}$, mean period $\bar{T}$ and persistence $P$. Among them the two measures $\bar{A}$ and $\bar{T}$ have been used for description of electroencephalograms (eeg), and their meaning is so clear that there is no need to add any explanation, but the other measure $P$ has been newly introduced by one of the authors, and so there are many points to be elucidated, especially with regard to its theoretical meaning and applicability in practice.

## 1. The Law of Amplitude.

In order to understand the meaning of the persistence, we must know the law of amplitude after which individual waves appear. Motokawa and Mita[2] have shown that the $\alpha$-waves of man vary their amplitude from moment to moment according to a very simple statistical law:

$$w = 2h^2 A e^{-h^2A^2} dA, \qquad (1)$$

where $w$ is the probability that the amplitude lies between $A$ and $A+dA$, $h$ being a constant characteristic of the given eeg.

This law holds for any sort of brain-waves, e.g. for the $\alpha$-waves of man, $\delta$-waves of epileptic patients, the main rhythm of various animals etc., provided that the periods of individual waves are not too greatly different from one another.

The true mean amplitude $\tilde{A}$ by which the arithmetic mean of infinitely great number of waves is meant can be obtained from the law of amplitude in the following manner:

$$\tilde{A} = \int_0^\infty A 2h^2 A e^{-h^2A^2} dA = 2h^2 \int_0^\infty A^2 e^{-h^2A^2} dA = \frac{\sqrt{\pi}}{2h}. \qquad (2)$$

## 2. The Persistence of Brain-Waves.

The law of amplitude predicts how frequently the wave of a given amplitude will appear in an eeg of sufficient length, but can tell us nothing about the order of appearance of waves.

As far as the frequency of appearance is concerned the following two series 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. and 5, 6, 7, 3, 2, 8, 10, 9, 4, 1. are equivalent to each other, but they are quite different with respect to the order of appearance. The order of appearance is a very important factor determining the pattern of an eeg, and the persistence $P$ is a measure chosen to express this property quantitatively. $P$ is defined as the mean of the number of waves which are greater than $\bar{A}/2$ and follow one after another without cessation. If waves greater than $\bar{A}/2$ and those smaller than it are expressed by the marks ○ and × respectively, the persistence of the eeg in which both sorts of waves are distributed in the following order:

$$\times\times \underbrace{\bigcirc\bigcirc\bigcirc}_{3} \times\times \underbrace{\bigcirc\bigcirc}_{2} \times\times\times\times \underbrace{\bigcirc\bigcirc\bigcirc\bigcirc\bigcirc}_{5} \times\times \underbrace{\bigcirc}_{1} \times \underbrace{\bigcirc\bigcirc\bigcirc}_{3} \times\times$$

must be equal to $(3+2+5+1+3)/5=2.8$. Generally, the persistence equals the total number of waves greater than $\bar{A}/2$ divided by the number of sequences of such waves. The measurement of persistence is carried out in the following way. In the first place, the mean amplitude $\bar{A}$ is obtained by means of Motokawa's method[1] which is very convenient to use and yields comparatively accurate results. After the determination of $\bar{A}$, the amplitudes of waves are compared with the distance between the two sharp points of a divider taken equal to $\bar{A}/2$ one after another, to see how many successive waves remain greater than $\bar{A}/2$. The number of waves of such a sequence is noted down, and the same procedure is repeated up to the end of the eeg. The so noted numbers are added together and divided by the number of sequences to obtain persistence.

The value of persistence is, in general, different from record to record even if they are taken from one and the same subject under the same external conditions. Fig. 1 shows the frequency-distribution of $P$ at six normal subjects. For the construction of each histogram 50–250 records were used, each of which contained about 200 $\alpha$-waves.

The range of variation seems to be much wider for $P$ than for any other measures like $\bar{A}$ and $\bar{T}$. We know that the greatest variation of $\bar{T}$ is about 10%, and that $\bar{A}$ is less stable than $\bar{T}$, but not so unstable as $P$. To get the idea of the error in the measurement of $P$, we measured the persistence of one and the same record repeatedly twenty times and found the variance $\frac{1}{N}\sum_{i=1}^{N}(P_i-\bar{P})^2$ to be as small as 0.059. This value is obviously too small to account for the marked fluctuations of $P$ shown in Fig. 1.

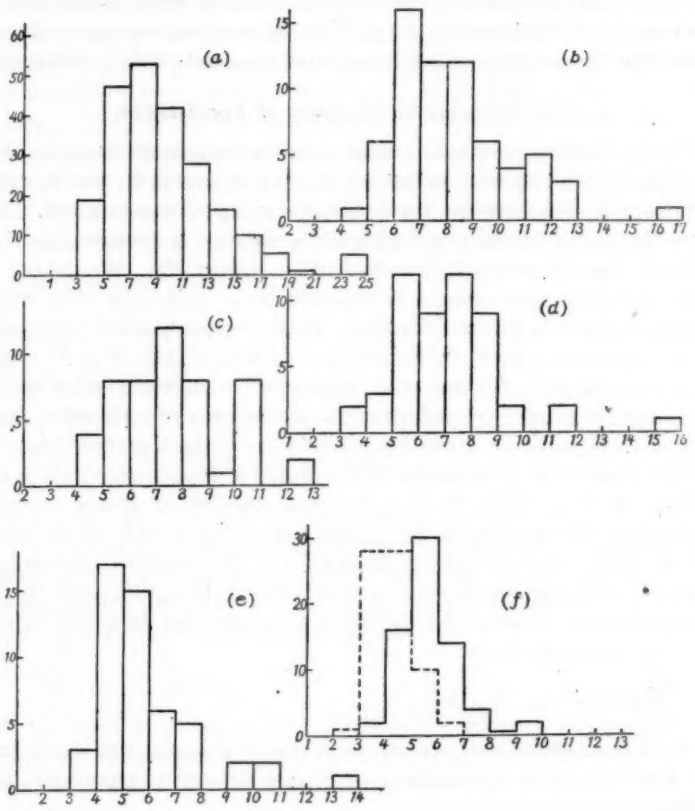


Fig. 1. Frequency-distribution (histograms) of the persistence of eeg of six normal subjects. Abscissa: Persistence, Ordinate: Frequency of appearance. (f): Histogram for the standard passive state of consciousness (full line) and that in sensory excitation (broken line).

The values of the variance of $P$ are as large as 20.7, 4.44, 4.07, 4.24 and 3.837 for (a), (b), (c), (d) and (e) in Fig. 1 respectively.

These marked fluctuations of $P$ may lessen the reliability of persistence as a statistical measure of eeg, but it can be said that the greater variability of $P$ might be due to its greater sensibility to delicate changes in the functional state of the brain. We cannot remain in the same state of consciousness even under constant external conditions, and such delicate changes in the brain might express themselves in the fluctuations of $P$. We sometimes obtained eeg of very different pattern from one and the same subject,

and in such cases the persistence of eeg was found to be very different from one another, the differences in $\bar{A}$ and $\bar{T}$ being not always so marked. This suggests that the persistence can be a better measure of the pattern of eeg.

### 3. The Theoretical Meaning of Persistence.

The persistence has its own value as an experimental measure suitable for the quantitative description of eeg, but it is desirable for the interpretation of data to elucidate the theoretical meaning of this measure.

We designate the state in which the amplitude is greater than $\bar{A}/2$ as $n$, and the state in which it is smaller than $\bar{A}/2$ as $\mathcal{N}n$. We denote with $W(n, n)$ the probability that $n$ is followed by $n$, and with $W(n, \mathcal{N}n)$ the probability that $n$ is followed by $\mathcal{N}n$. Then, the probability that $n$ occurs twice in succession is given by the product of $W(n, n)$ and $W(n, \mathcal{N}n)$, that is $W^{2-1}(n, n)\, W(n, \mathcal{N}n)$, because two successive occurrences of $n$ and appearance of $\mathcal{N}n$ after $n$ are independent phenomena of each other, and so the probability must be given by the product of both probabilities. For the same reason, the probability that $n$ occurs $k$ times successively is equal to $W^{k-1}(n, n)\, W(n, \mathcal{N}n)$. If $\mathcal{N}$ is the total number of groups containing only $n$-states, the number of the groups containing $k$ such waves must be equal to $\mathcal{N}W^{k-1}(n, n)W(n,\mathcal{N}n)$. Hence, the total number of waves contained in such groups is equal to $k\mathcal{N}W^{k-1}(n,n)W(n,\mathcal{N}n)$, and the total number of waves contained in all sorts of groups can be obtained as the sum of the following series:

$$\sum_{k=0}^{\infty} k\mathcal{N}W^{k-1}(n,n)\,W(n,\mathcal{N}n).$$

From the definition of persistence as the total number of waves greater than $\bar{A}/2$ divided by the number of groups follows at once such an expression of $P$ as:

$$P=\frac{1}{\mathcal{N}}\sum_{k=0}^{\infty} k\mathcal{N}W^{k-1}(n,n)\; W(n,\mathcal{N}n)=\sum_{k=0}^{\infty} kW^{k-1}(n,n)\,W(n,\mathcal{N}n)$$
$$=W(n,\mathcal{N}n)\;[1+2W(n,n)+3W^{2}(n,n)+4W^{3}(n,n)+\;\ldots\ldots\ldots\ldots].$$

The infinite series in the parenthesis reduces to $1/[1-W(n,n)]^2$ by simple calculation of the sum of the series, taking account of the relation $W(n,n)<1$.

Since $W(n,\mathcal{N}n)$ is the probability that $n$ is followed by $\mathcal{N}n$, it must be equal to $1-W(n,n)$, so that $P$ is expressed in terms of $W(n,n)$ or $W(n,\mathcal{N}n)$ as follows:

$$P=\frac{1}{1-W(n,n)}=\frac{1}{W(n,\mathcal{N}n)}. \qquad (3)$$

As seen from this relation, the persistence is the reciprocal of the probability that a wave greater than $\bar{A}/2$ is followed by a wave smaller than $\bar{A}/2$.

It is important from the theoretical as well as practical point of view to evaluate the persistence for the ideal case in which all the waves are independent of one another. In such an ideal case the probability $W(n,n)$ that $n$ is followed by $n$, must be equal to the probability for the independent occurrence of $n$, which will be denoted by $W(n)$ in the following. $W(n)$ can be obtained from the law of amplitude (1), for it gives the probability for the independent occurrence of a wave of any amplitude. The probability for the appearance of all the waves greater than $\bar{A}/2$ can be obtained by integrating (1) from $\bar{A}/2$ to infinite as follows:

$$W(n)=2h^2\int_{\bar{A}/2}^{\infty} Ae^{-h^2A^2}dA=e^{-\frac{1}{4}h^2\bar{A}^2}. \tag{4}$$

By eliminating $h$ from (4) and (2) we obtain

$$W(n)=e^{-\frac{\pi}{16}\left(\frac{\bar{A}}{\bar{A}}\right)^2}, \tag{5}$$

and consequently

$$P=\frac{1}{1-W(n)}=\frac{1}{1-exp.\left[-\frac{\pi}{16}\left(\frac{\bar{A}}{\bar{A}}\right)^2\right]}. \tag{6}$$

This relation indicates that the persistence depends upon the deviation of the measured mean amplitude $\bar{A}$ from the true mean amplitude $\bar{A}$. If a sufficiently great number of waves is taken for the measurement of the mean amplitude the ratio of $\bar{A}$ to $\bar{A}$ can be put equal to unity, and the persistence reduces to a constant value 5.6, since

$$\lim_{\bar{A}\to\bar{A}} P=\frac{1}{1-exp.\left(-\frac{\pi}{16}\right)}=5.6.$$

When the number of waves is not sufficiently great as is the case in our experiments, the persistence can fluctuate owing to the fluctuation of $\bar{A}$. But the fluctuation of $P$ of real eeg cannot entirely be due to the fluctuation of the measured mean amplitude. The latter is nothing but one of many factors influencing the persistence. If the fluctuation of the measured mean amplitude were the only factor involved, the value of $P$ of real eeg should fluctuate round the theoretical value 5.6. As seen from Fig. 1, the most frequently appearing value of $P$ are in general greater than the ideal value 5.6. This fact indicates that the assumption that all the waves are independent of one another does not hold good for real brain waves. The real brain waves, therefore, must be dependent of one another, at least, in such a manner that the preceding wave has some after-effect on the succeeding one. If the after-effect facilitates the appearance of a wave of the same sort the persistence must be greater than that in the ideal case.

### 4. The Persistence of a Wave-Complex.

In some cases the maximum of the histogram of $P$ lay at a smaller value than 5.6, as seen from (e) in Fig. 1. Such small values of $P$ are usually obtained in the excited state of the brain. A decrease of $P$ can easily be realized by stimulating the subject in some way or other.

The effect of visual stimulation upon the persistence is illustrated in (f) of Fig. 1; the histogram in full line is for a subject with his eyes closed, and that in broken line is for the same subject with his eyes open. The histogram shifts as a whole to the left on visual stimulation, indicating that the persistence is reduced by sensory excitation.

Such a decrease of $P$ far below the theoretical value 5.6 could be explained by assuming an inhibitory after-effect, but we feel some difficulty to answer the question why the after-effect is usually facilitation, but in excited states inhibition. Another explanation of small values of $P$ in the excited states would be found in the composite nature of eeg taken under these conditions.

Eeg in the excited states are generally composed of sequences of waves which are too different from one another to regard as belonging to one and the same population, and such eeg yield usually very small values of $P$.

The ideal value of $P$, 5.6 has been obtained from the theory of a single population, and it is this very theory that has necessitated the assumption of an inhibitory after-effect in order to explain the appearance of smaller values of $P$ than 5.6. However, it is inadequate at least for eeg in the excited states to rely upon this way of explanation, because they consist of obviously different sorts of waves, e.g. $\alpha$- and $\beta$-waves which belong apparently to different populations. These two sorts of waves may co-exist in any eeg obtained under the standard condition of consciousness or in the passive state of the mind, but we usually measure only the predominating $\alpha$-waves, neglecting the $\beta$-waves superposed upon them. Hence, it is justified to regard such a standard eeg as a single population of waves. On the contrary, an eeg taken in excited states consists usually of various sorts of waves arranged in such a manner that some portions of the eeg are covered by a certain kind of waves, but other portions by other sorts of waves. Such an eeg consisting of two or more populations may be designated a wave-complex.

We can prove that the persistence of a wave-complex is always smaller than that of the waves of a single population. Suppose that an eeg be composed of two kinds of waves $x$ and $y$, the mean amplitudes of which are $\bar{A}_x$ and $\bar{A}_y$ respectively, and that the probability for the appearance of $x$ and $y$ in the eeg be $m$ and $n$ respectively, then the mean amplitude of the eeg $\bar{A}$ is given by a linear combination of $\bar{A}_x$ and $\bar{A}_y$ as follows:

$$\bar{A}=m\bar{A}_x+n\bar{A}_y. \qquad (7)$$

When $x$ and $y$ appear alternatively in the eeg the sum of the probabilities for appearance of both sorts of waves must be equal to unity, namely:

$$m+n=1. \qquad (8)$$

It will be shown in the following that the persistence of this eeg is smaller than that of any eeg of a single population $x$ or $y$. For simplicity, let the waves be independent of one another or free from an after-effect, then the probability that any wave of the $x$-population is greater than $\bar{A}/2$ is $\exp.\left[-\frac{\pi}{16}\left(\frac{\bar{A}}{\bar{A}_x}\right)^2\right]$ after the relation (5). Since the probability for the appearance of a wave of the $x$-population is $m$, the product of these two probabilities gives that for the appearance of any $x$-wave which is greater than $\bar{A}/2$. Similarly the probability for the appearance of any $y$-wave which is greater than $\bar{A}/2$ must be $n\ \exp.\left[-\frac{\pi}{16}\left(\frac{\bar{A}}{\bar{A}_y}\right)^2\right]$. Hence the total probability that any wave is greater than $\bar{A}/2$ is given by the sum of the probabilities for both populations, namely:

$$W=m\ \exp.\left[-\frac{\pi}{16}\left(\frac{\bar{A}}{\bar{A}_x}\right)^2\right]+n\ \exp.\left[-\frac{\pi}{16}\left(\frac{\bar{A}}{\bar{A}_y}\right)^2\right], \qquad (9)$$

TABLE I.

The persistence of an eeg consisting of two kinds of waves $x$ and $y$ whose mean amplitudes are $\bar{A}_x$ and $\bar{A}_y$ respectively. The probability of appearance in the eeg for both sorts of waves were assumed to be equal to each other. The statistical after-effect was neglected. $W$ is the probability for the appearance of waves greater than one half mean amplitude of the eeg.

| $\bar{A}_x:\bar{A}_y$ | $W$ | $P$ |
|---|---|---|
| 1 | 0.882 | 5.6 |
| 2 | 0.804 | 5.1. |
| 4 | 0.749 | 3.98 |
| 6 | 0.698 | 3.33 |
| 8 | 0.655 | 3.1 |
| 10 | 0.619 | 2.62 |

TABLE II.

The persistence of a wave-complex. $m$ and $n$ represent the probability for the apppearance of any $x$-wave and of any $y$-wave respectively. The ratio $\bar{A}_x:\bar{A}_y$ was assumed equal to 1:4. $W$ is the probability for the appearance of waves greater than one half mean amplitude of the wave-complex.

| $m:n$ | $W$ | $P$ |
|---|---|---|
| 0 : 1 | 0.822 | 5.60 |
| 0.1: 0.9 | 0.799 | 4.98 |
| 0.2: 0.8 | 0.779 | 4.53 |
| 0.3: 0.7 | 0.765 | 4.29 |
| 0.4: 0.6 | 0.754 | 4.06 |
| 0.5: 0.5 | 0.749 | 3.98 |
| 0.6: 0.4 | 0.749 | 3.98 |
| 0.7: 0.3 | 0.756 | 4.09 |
| 0.8; 0.2 | 0.770 | 4.34 |
| 0.9: 0.1 | 0.791 | 4.78 |
| 1 : 0 | 0.882 | 5.60 |

and the persistence of the eeg in question can be evaluated according to the formula $P=1/1-W$, and the relations (7), (8) and (9), if the rations $m:n$ and $\bar{A}_x:\bar{A}_y$ are given.

It will be numerically proven that the persistence so calculated is smaller than the value for a single population 5.6. Table I shows how the persistence is altered as the ratio $\bar{A}_x:\bar{A}_y$ changes from 1 to 10 when the ratio $m:n$ is fixed to a constant value 1. From this table it can be seen that the persistence of a wave-complex is always smaller than that of waves of a single population, and that the greater the difference in the mean amplitude of the component wave-populations, the smaller the value of persistence.

Table II shows the effect of the ratio $m:n$ on the persistence when the ratio $\bar{A}_x:\bar{A}_y$ is fixed to a constant value 1:4. It is to be noted that $P$ is minimum when two sorts of waves appear with the same probability. In general, the persistence is least when two sorts of waves are greatly different in their amplitude and mixed in equal proportion.

The above argument applies also to cases in which more than two sorts of waves are involved. We have already shown that the persistence is generally small in the excited state of the brain. In normal man, small values of $P$ are in most cases accompanied by small values of the mean amplitude. This is clearly caused by $\beta$-waves which appear frequently in the excited state. We studied the correlation between persistence and mean amplitude on 63 normal subjects and found 0.43 as the correlation-coefficient. This positive correlation is evidently due to dominant $\beta$-waves which cause both $P$ and $\bar{A}$ to decrease simultaneously.

As the $\beta$-waves are a sign of cerebral excitation, a small value of $P$ associated with a small value of $\bar{A}$ is to be regarded as an indication of a high excitation-level of the brain. A small value of $P$ alone, however, does not always indicates any cerebral excitation, for a decrease of $P$ ensues from the appearance of $\delta$-waves which are no sign of cerebral excitation. In such cases a small value of $P$ is accompanied by a large value of $\bar{A}$, because the $\delta$-waves usually have great amplitudes. A good example of such cases is the eeg of epileptic patients in the interval between seizures. In Table III mean values of $P$ at five normal subjects and those at five epileptic patients are shown. The mean values are in all cases but one of the normal subjects greater than the ideal value 5.6, whereas it is smaller in all cases of epileptic patients. The exceptional case was a $\beta$-rich eeg with a small mean amplitude suggesting the high cerebral excitation-level of the subject.

It has been shown that in the ideal case of a single population without any after-effect the persistence is wholly determined by the mean amplitude. Moreover, there exists some positive correlation between per-

TABLE III.

Mean values of persistence $\bar{P}$ in 5 normal subjects and 5 epileptic patients. $n$: number of records measured for each subject.

| Normal | | Epileptic | |
|---|---|---|---|
| $\bar{P}$ | $n$ | $\bar{P}$ | $n$ |
| 9.5 | 226 | 3.79 | 38 |
| 8.24 | 66 | 4.92 | 47 |
| 7.65 | 51 | 5.31 | 22 |
| 7.1 | 52 | 3.72 | 22 |
| 4.98 | 51 | 3.87 | 38 |

sistence and mean amplitude. These circumstances might make doubtful whether the persistence can be an independent statistical measure. But in reality, both measures $P$ and $\bar{A}$ go parallel in some cases, however, vary independently in other cases, so that the one cannot be represented by the other. It must be emphasized that at least three statistical measures, $\bar{T}$, $\bar{A}$ and $P$ are necessary to characterize eeg numerically.

SUMMARY.

It was proposed to analyse electroencephalograms (eeg) and to express them quantitatively with three statistical measures, mean amplitude $\bar{A}$, mean period $\bar{T}$ and persistence $P$. The persistence is the mean of the number of waves greater than $\bar{A}/2$ following successively without interruption.

1. 50–250 eeg were taken at each subject and analysed. The histogram of $P$ had its maximum at the value of $P$ 4–8, and the mean values of $P$ were 9.5, 8.24, 7.65, 7.1 and 4.98 in the standard passive state of consciousness of normal subjects.

2. The theoretical meaning of persistence was discussed. The value of $P$ 5.6 was calculated from the theory for the ideal eeg in which all waves are independent of one another and belong to one and the same population of waves.

3. In general, the persistence is greater than 5.6, and this relation was regarded as expression of dependence among individual waves.

4. The persistence is smaller than 5.6 in cerebral excitation and under other conditions that more than a single sort of waves appear in eeg. It was theoretically proven that the persistence can decrease below the ideal value 5.6 by mixing more than one sort of waves.

5. Mixing of $\beta$-waves with normal $\alpha$-waves reduces both $P$ and $\bar{A}$, whereas mixing of $\delta$-waves with normal $\alpha$-waves decreases $P$, but increases $\bar{A}$.

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# Über die bakterizide Wirkung von Guanidinderivaten, welche bei uns dargestellt worden sind.

Von

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Schon im Jahre 1927 waren von Kumagai, Shikinami und Kawai verschiedene Polymethylendiguanidine, bei denen je ein freies Guanidinmolekül an den beiden Enden von 4 bis 10 Methylenketten vorhanden war, synthetisch dargestellt worden. Unter diesen Guanidinderivaten riefen Okta- und Dekamethylendiguanidin bei Diabetikern einen gewissen Glykosuriehemmenden Effekt hervor.

In 1940 synthesierten Marshall, Bratton, White und Litchfield Sulfanilylguanidin, bei dem ein Guanidinmolekül an der Sulfongruppe des Sulfanilin gebunden war. Das Mittel ist auf die Behandlung der bakteriellen Darmkrankheiten angewandt worden.

Es ist hierauf von 1947 her bei uns über die bakterizide Wirkung unserer Polymethylendiguanidine im Vergleich mit der des Sulfanilylguanidins beobachtet worden. Diese Ergebnisse sind folgendermassen kurzgefasst mitgeteilt.

Bei unseren Polymethylendiguanidinen z.B. bei Äthylen-, Tetra-, Hexa-, Okta- und Dekamethylendiguanidin nimmt die bakterizide Wirkung Hand in Hand mit der Verlängerung der Methylenkette zu, und bei Oktamethylendiguanidin erreicht den Gipfelpunkt.

Hinsichtlich der bakteriziden Wirkung gegen Shigella, Salmonella, Colibazillen, Staphylokokken und Enterokokken sind Hexa-, Okta- und Dekamethylendiguanidin einige zehnfach stärker als Sulfanilylguanidun. Bei Ruhrkranke genügt perorale Applikation des Okta- und Dekamethylendiguanidins in einer Menge von 15 bis 20 mg eine gute Folge zu bringen.

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# Biochemical Studies on Carbohydrates.

## CIX. Amino Sugar in Osseomucoid-Glycidamin.

By

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In continuation of the work on the amino sugar of chondromucoid-glycidamin,[1] the present subject of investigation was taken up. The glycidamin in osseomucoid was separated by dissolving the latter by means of $Ca(OH)_2$ and following shaking with an amylalcohol-chloroform mixture. After its hydrolysis, the amino sugar was isolated in the form of hydrochloride as before and identified with glucosamine from the behaviour of the product on heating and by qualitative and quantitative analyses and measurement of optical rotation.

### Experimental.

### Separation of the Glycidamin Moiety from Osseomucoid and Its Composition.

To give a typical example:— 5.0 gm. of osseomucoid prepared as was by Hisamura[2] were dissolved in 200 cc. of water by the aid of the saturated $Ca(OH)_2$, avoiding the risk of shifting the pH of the solution beyond neutral. Some insoluble part was centrifuged off. The supernatant yellowish fluid was shaken thoroughly with 1/5 the volume of an amylalcohol-chloroform mixture (1:3 by volume) and centrifuged. The watery layer was poured out and treated in a like manner, having supplemented $Ca(OH)_2$ to maintain its pH at neutral. When no more jelly was formed after repetition of the processes, the centrifuge tubes containing the jelly were tapped, the separating organic mixture decanted off and the remainders were collected together by means of alcohol. The jelly was shrunk by alcohol. It was further washed with alcohol and ether and dried. Once again treated with the organic mixture similar to above, the recovered portion was free from chondroitinsulfuric acid entirely (glucuronic acid was not detected by Neuberg and Saneyoshi method). It was then

washed with HCl-containing 95% alcohol three times and with plain alcohol and ether in sequence and dried. Yield, 3.1 gm. This material was used for separating the amino sugar (next section).

On the other hand, to analyze the glycidamin, 1.1 gm. of osseomucoid was freed from chondroitinsulfuric acid as above but further treated with formamide as follows. Coagulation with the amylalcohol-chloroform mixture was carried out three times and immediately to the powder, which had not been as yet removed from calcium, (this amounted to 0.33 gm.) were added 4 cc. of formamide drop by drop with kneading. After prolonged centrifugation, some insoluble part separated was again agitated with formamide (2 cc.) and centrifuged. The supernatant fluids were united and precipitated with alcohol and the precipitates were washed with alcohol and ether and dried in a desiccator. 0.29 gm. was yielded. The product gave intensive biuret, Molisch and Hiyama[3] reactions. Liebermann and Osaki-Turumi (both the direct and indirect) tests also resulted in positive.

It analyzed as is demonstrated in Table I.

TABLE I.

Composition of Osseomucoid-Glycidamin.

| Analysis | In per cent | In equivalents per equivalent weight |
|---|---|---|
| Nitrogen | 13.5 | |
| Amino sugar | 6.35 | 1.0 |
| Non-amino sugar | | |
| Indole r. | 6.45 | |
| Orcinol r. | 6.39 | |
| α-Naphthol r. I | 6.23 | |
| Mean | 6.36 | 0.99 |
| Hydrolysable sulfur | 0.52 | 0.46 |
| Ash | 0.93 | |

## Isolation of the Amino Sugar as Hydrochloride from the Hydrolysate of the Osseomucoid-Glycidamin.

3 gm. of the first batch of the glycidamin were taken for the use. Hydrolysis and fractionation of the hydrolysate were performed as in the case of chondromucoid-glycidamin.[1] The fraction exhausted with butanol was also likewise dissolved in a hydrochloric acid-alcohol mixture and precipitated carefully with acetone, but, probably due to the poor hexosamine content of the glycidamin, the deposits consisted largely of oil and small irregular crystals came out only in few round assemblages. When no more precipitation occurred by increasing acetone (added from time to time during 2 weeks), the mother fluid was decanted and the deposits

dissolved again in a mixture of HCl (20%) and alcohol and precipitated by addition of acetone in small portions as before. On another repetition of the operations an all-crystalline deposit (trigonic plates) appeared. The crop was no more than 17 mgm. The oily residue after evaporation of the decanted mother liquids could not be changed crystalline, notwithstanding the same technique was adapted. The butanol extract showed the positive Elson and Morgan's test for hexosamine (not intensive), but attempts to separate it failed:—

1. No crystals came out from the thick condensate of the extract (the extract was distilled at a low temperature) on standing in an ice-chest.

2. This condensate was further dried up and after dissolving in some water, agitated with an excess of 25% mercuric acetate solution. The filtrate of the precipitates was freed from Hg and acetic acid with $H_2S$ and ether respectively and concentrated to a syrup under diminished pressure. This was taken up in 20% HCl and diluted with alcohol and acetone added, as often tried. But an only oily deposit was obtained.

3. The oil (250 mgm.) was treated with 2-hydroxynaphthaldehyde by the method of Jolles and Morgan[4] to derive the Schiff's base from the amino sugar. Nothing but a thick-mucilaginous substance was given.

4. This was hydrolyzed with 4 n. HCl to recover the amino sugar as hydrochloride. The hydrolysate was condensed, after extracting off of the liberated aldehyde, and the remaining syrup was dissolved in alcohol and precipitated by adding acetone. The oily deposit became crystalline gradually. Nevertheless the amount was so minute that separation of them was abandoned.

### Properties of the Isolated Hexosamine Hydrochloride.

The crystals separated above gave the positive hexosamine test of Elson and Morgan and chloride test and reduced hot Fehling solution. On heating, the substance began charring at about 180°C but did not melt even at 280°C. Its optical rotation in water is illustrated in Table II. Extrapolation gave the initial $[\alpha]_D$ of $+101°$.

N (micro Kjeldahl method).

4.08 mgm. substance, 1.864 cc. 0.01 n. acid neutral. by $NH_3$.

$C_6H_{13}O_5N \cdot HCl$. Calc., 6.50%; found, 6.40%.

Thus all the findings indicated the product to be $\alpha$-glucosamine hydrochloride.

TABLE II.

Optical Rotation in Water of the Hexosamine Hydrochloride from Osseomucoid-Glycidamin.

| Time after dissolution min. | $\alpha$ deg. | $[\alpha]_D = \frac{\alpha \times 100}{1.223 \times 1}$ deg. |
|---|---|---|
| 4 | +1.21 (8°C) | +98.9 |
| 15 | +1.14 ( 〃 ) | +93.2 |
| 30 | +1.10 ( 〃 ) | +89.9 |
| 60 | +1.04 ( 〃 ) | +85.0 |
| 60×2 | +0.98 ( 〃 ) | +80.1 |
| 60×4 | +0.91 ( 〃 ) | +74.4 |
| 60×6 | +0.89 (7°C) | +72.8 |
| 60×24 | +0.89 ( 〃 ) | +72.8 |

The expenses of this work were defrayed by a grant given from the Education Department through the Grant Committee of the National Research Council. H. Masamune.

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3) Hiyama, Tohoku J. Exp. Med., in press.
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# Biochemical Studies on Carbohydrates.

## CX. Hexosamine Compounds in Lung.

First Report.

By

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(Received for publication, August 3, 1947)

From pig lung have been isolated two glycidamins besides heparin. The present account deals with the preparation procedure and the results of chemical and biological tests.

## Experimental.

### Preparation Procedure and Chemical Properties of the Products.

Fresh pig lungs were removed mechanically from bronchioles and the adhering pleural membrane as perfectly as possible, washed from blood stains and minced. The tissue pulp was placed in 5 volumes of 95% alcohol for several days, squeezed and again dehydrated with 3 volumes of the alcohol similarly. It was followingly boiled twice with 10 volumes of the alcohol for 2 hours and dried in a Faust apparatus. 500 gm. of the dried material obtained thus from 2500 gm. of the fresh pulp were then agitated in 5 volumes of 5% NaCl solution for 2 days, using thymol (0.5 cc. of its 25% alcoholic solution for every 100 cc. of the NaCl solution) as a preservative. The extract was percolated and centrifuged. The turbid supernatant liquid were precipitated[1] by acidifying to pH 4.5 with 30% acetic acid. Centrifuged again. The clear liquid obtained was neutralized with 2% NaOH solution, dialyzed (cellophane membrane) against running water for 3 days, and some precipitate, which appeared thereby, having been removed, distilled under reduced pressure to about 10 cc. 3 volumes of 95% alcohol and a little sodium acetate were added and the deposit washed with 95% alcohol and ether and dried in a vacuum desiccator ($CaCl_2$). 0.9 gm. was the yield. A repeated extraction of the residue gave 0.4 gm.[2]

The products combined were taken up in 10 cc. of water, and a small part undissolved was centrifuged off, then the liquid was acidulated, removed from the acid-insoluble substance[3] and precipitated after neutralization as above. The dried substance weighed 1.1 gm. and was positive in biuret, Molisch (violet), aniline acetate (for pentose and hexuronic acid), Neuberg and Saneyoshi naphthoresorcinol (for hexuronic acid), Osaki and Turumi (both by the direct and indirect processes), barium sulfate (for hydrolysable sulfur) and ammonium molybdate (for phosphorus) tests, which suggested our having a mixture of proteins, nucleic acid, heparin and other hexosamine compounds. Hence, removal of the usual proteins (made up of almost merely amino acids) was carried out prior to fractionation as follows. The substance was at first kneaded with a little glycol to a dough, stirred up with the solvent that had been increased to 10 cc. and subjected to prolonged centrifugation. The transparent supernatant fluid was precipitated with 2 volumes of acetone and a little sodium acetate, and the precipitate was washed with acetone (three times) and ether (twice) and dried in vacuo over $CaCl_2$. 0.7 gm. of white powder was obtained. Extraction[4] made again with 5 cc. of glycol gave a 0.17 gm. crop.

Preliminary fractionation. The whole product was made into a thick paste with a little water ($<$1 cc.) and precipitated with 5 cc. of glacial acetic acid (99%). Centrifuged. The centrifugate was freed from acetic acid by washing with acetone (4 times) and dried after substituting ether for acetone (3 changes of ether were used). On repetition of these processes phosphorus was eliminated by and by and the final phosphorus-free substance (white powder; obtained after 5 reprecipitations) amounted to 0.5 gm.—Fr. A. The supernatant liquids were united and mixed with 2 volumes of acetone and the deposit washed and dried again similar to above. 0.3 gm. (yellowish powder) was yielded here.—Fr. B. The results of qualitative tests are listed in Table I, wherefrom the transition of nucleic acid and a probable glycidamin or glycidamins into Fr. B. looks to have occurred.

Subdivision of Fr. A. This fraction (0.5 gm.) was kneaded with 2 cc. of water, the dough weakly alkalized with drops of 2% NaOH (to pH 7.2–7.4) and 5 cc. of 2% picric acid added to precipitate the solution. After centrifugation, the centrifugate, which was biuret-positive and gave no positive Osaki-Turumi test both by the direct and indirect processes, was discarded and the clear supernatant fluid was precipitated with acetone (2 volumes). The precipitate was treated first with cold acetone (three times) and then with the boiling (three times), whereby picric acid was entirely washed off, and dried up in vacuo. It was next dissolved in 20 cc. of water (dissolved clearly), and after neutralization, shaken many times

TABLE I.

| Test | Fr. A | Fr. B |
|---|---|---|
| Biuret | + | + |
| α-Naphthol-$H_2SO_4$ | Violet (Molisch, +) | Blood red with violet tint |
| Aniline acetate (for pentose and hexuronic acid) | + | + |
| Neuberg-Saneyoshi naphtho-resorcinol | + | − |
| Osaki-Turumi | | |
| Direct | + | + |
| Indirect | + | + |
| $BaSO_4$ (for hydrolysable S) | + | − |
| Ammonium molybdate (for P) | − | + |
| Ammoniacal $AgNO_3$ (for purine bases) | − | + |

with chloroform (at first with 5 cc. and afterwards with 2 cc.), each time the gel formed being separated by centrifugation. When no more gel came out, the solution was acidified to pH 2.0 (with 2% HCl), and deproteinization was continued using 2 cc. portions of chloroform. Finally the watery layer[5] was mixed thoroughly with 5 gm. of kaolin, and filtered under suction, then the filtrate neutralized (with 2% NaOH), distilled (under reduced pressure) to about 1 cc. and precipitated with acetone (3 volumes). The precipitate was washed with acetone and ether and dried. 0.1 gm. of white powder was yielded.—Fr. $A_{1a}$. Kaolin on the funnel was suspended in 30 cc. of water, neutralized (with 2% NaOH) and again filtered off on a Büchner funnel. The filtrate here was also condensed and deposited as the former. Crop, 30 mgm.—$A_{1b}$. As is demonstrated in Table II, not only Fr. $A_{1a}$ but also $A_{1b}$ seemed heparin, although the latter was contaminated with a trace of glycidamin or of an acetyl-hexosamine-containing carbohydrate.

TABLE II.

| Test | Fr. $A_{1a}$ | Fr. $A_{1b}$ |
|---|---|---|
| Biuret | − | − |
| α-Naphthol-$H_2SO_4$ | Green (Goldschmiedt, +) | Green (Goldschmiedt, +)* |
| Aniline acetate | + | + |
| Neuberg-Saneyoshi | + | + |
| Osaki-Turumi | | |
| Direct | − | − |
| Indirect | − | ± |
| $BaSO_4$ (for hydrolysable S) | + | + |

* A faint red ring appeared neighbouring the green at the surface of contact of the two layers, and on shaking together the mixture changed light violet.

The jellies from the neutral and acid solutions were combined, washed with water, acidified to pH 2 (with HCl), 3 times and shrunk by means of 95% alcohol. Washed with alcohol (95%) and ether and dried. Yield, 0.15 gm.—Fr. $A_2$. This substance was soluble in water at neutral and alkaline reactions and insoluble at pH's of 4.0 and less. Tests indicated it to be a glycidamin (TAable III).

TABLE III.

| Test | Fr. $A_2$ |
|---|---|
| Biuret | + |
| α-Naphthol-$H_2SO_4$ | Violet (Molisch, +) |
| Neuberg-Saneyoshi | − |
| Osaki-Turumi | |
| Direct | + |
| Indirect | + |
| $BaSO_4$ (for hydrolysable S) | + |

Elimination of nucleic acid from Fr. B. 0.15 gm. of this fraction was dissolved in 4 cc. of water. To the solution, which was faintly acid (pH, 4.5), was added the saturated $Ba(OH)_2$ in drops first until neutral and then to pH 9.4, the precipitate appearing at each point being centrifuged off. The second supernatant liquid was acidified back to pH 2.0 with 3% HCl and subjected to a similar procedure over again[6]. Barium was removed with 3% $H_2SO_4$ solution and the precipitate by 3 volumes of 95% alcohol was washed with alcohol (95%, three times) and ether (twice) and dried in a vacuum desiccator. 90 mgm. of yellowish white powder were gained—Fr. $B_1$. It was water-soluble and was a glycidamin, judging from the results of reactions listed in Table IV.

TABLE IV.

| Test | Fr. $B_1$ |
|---|---|
| Biuret | + |
| α-Naphthol-$H_2SO_4$ | Violet (Molisch, +) |
| Aniline acetate | − |
| Osaki-Turumi | |
| Direct | + |
| Indirect | ++ |
| Ammonium molybdate | − |

*Remark.* In the tissue residue, which had been exhaustively extracted with the saline solution, was still detected hexosamine (analyzed by Hamasato and Akakura method). And on agitation with water an abundant amount of substances containing phosphorus and hexosamine came out in a fine suspended state. They could not be separated by centrifugation but could be filtered off on paper pulp by suction[7] and also subsided on addition of NaCl up to 5%[7] or of acetic acid. The precipitates only swelled without dissolution in alkaline ($Na_2CO_3$) water. It involved 4.5% hexosamine, but many re-depositions after taking up as a suspension in the alkaline water by means of HCl (added to pH of 2.0) as well as fractionation at different pH's neither raised the hexosamine content nor eliminated phosphorus. The nature of the hexosamine compound here has not been followed up.

Extracted with water, the residue showed no hexosamine reaction anymore.

## Biological Assays.

Hemopoietic and hemolytic activities, effect on blood coagulation and toxicity were examined.

Hemopoietic activity. Frs. $A_{1a}$ and $A_2$ showed to possess this property.

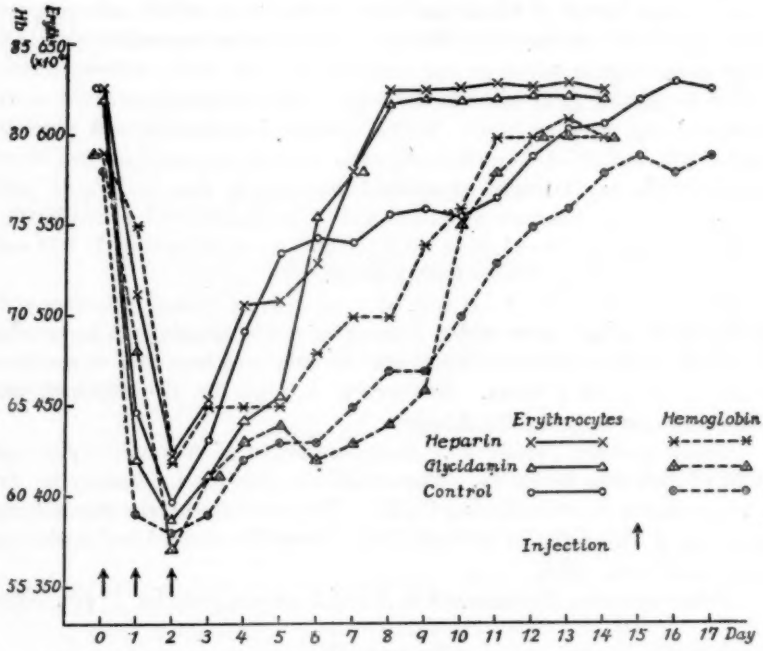


Fig. 1. Effect of heparin and the hemopoietic glycidamin from lung on the recovery of erythrocyte number and hemoglobin content of rabbits after bloodletting.

15 cc. of blood per kgm. bodyweight were drawn from an ear vein of rabbits and after 1/2, 24 and 48 hours, 1 mgm. of the substances per kgm. bodyweight was injected intravenously as dissolved in the physiological saline solution (1 mgm. in 1 cc.). Five rabbits were employed for each of the substances and for the control runs where plain physiological saline was injected. In the controls it took more than two weeks for both erythrocyte number (Thoma-Zeiss) and hemoglobin content (Sahli) to resume the values before the bloodletting (the mean values in three successive days), but in the cases where the substances were applied, 8–9 and 8–12 days

respectively were sufficient for the recovery. The curves plotted in Fig. 1 show the mean fluctuations of two experiments each. The experiments selected for the averaging were those on the rabbits all of which had closely resembling normal values of hemoglobin content and erythrocyte number.

Effect on blood clotting. The clotting time was measured with an apparatus similar in design to the one by Chargaff, Bancroft and Stanley-Brown.[8] Into the glass tubes (11×75 mm.) were pipetted 0.2 cc. of a solution diluted successively with physiological saline and then 0.5 cc. of human venous blood (B blood was used in this case) added, plunging the needle tip of the syringe to the bottom. The solution was substituted with simple physiological saline in one control and with 0.4% sodium citrate solution in physiological saline in the other. The temperature of the water thermostat was fixed at 20°C. In the controls coagulation took place in 5'20''–5'30'' and 22'–25' respectively after mixing the solution and blood together. Fr. $A_{1a}$ (heparin) prolonged the clotting time to 31', 11' and 6' respectively at dilutions of 1:200,000, 1:2,000,000 and 1:20,000,000. Fr. $B_1$ only doubled the clotting time (10'20'') at a dilution of 1:100 and Fr. $A_2$[9] had no effect on the clotting at all.

Hemolytic activity. To 0.2 cc. of a successively diluted solutions with physiological saline were added 2 drops of a 2% erythrocyte suspension (human B erythrocytes were taken) and the mixtures kept in a water thermostat at 37°C for 2 hours. Neither Fr. $A_2$[9] nor Fr. $B_1$ developed this power even at only 1:200 dilution.

Group specifity (Table V). Isoagglutination of A erythrocytes was inhibited distinctly by Fr. $B_1$. The inhibition exhibited minutely by Fr. $A_2$[9] is probably due to adhering Fr. $B_1$. The method was the one hitherto employed in this series of investigation. A and B sera used had agglutinin titers ×160 and ×80.

Poisonousness. 20 mgm. of Frs. A and B were applied for 10 gm. body-

TABLE V.

Occurrence of Isoagglutination of Erythrocytes, When the Sera are Preliminarily Treated with Fractions $A_2$ and $B_1$.

| Fraction | Dilution of Substance / Group of serum | 1% (Original) | ×10 | ×10² | ×10³ | ×10⁴ |
|---|---|---|---|---|---|---|
| $B_1$ | A | ++ | ++ | +++ | +++ | +++ |
| | B | − | − | − | + | ++ |
| $A_2$ | A | ++ | ++ | +++ | +++ | |
| | B | − | + | ++ | ++ | |

weight to a mouse intraperitoneally. But there appeared no poisoning symptom. For injection, Fr. B was dissolved immediately in physiological saline, but Fr. A was dissolved at first in water by addition of 5% $Na_2CO_3$ solution until neutral and an equal volume of 1.7% NaCl added.

## Summary.

1. Heparin and two glycidamins were isolated from the lung tissue of pig. One of the glycidamins contained hydrolysable sulfur.

2. Heparin and the hydrolysable sulfur-containing glycidamin proved hemopoietic.

3. The glycidamin devoid of hydrolysable sulfur inhibited isoagglutination of A erythrocytes specifically.

The costs of this work were covered by a grant by the Grant Committee for Scientific Researches of the Education Department, which is gratefully acknowledged.

## Bibliography and Notes.

1) The precipitate was small.

2) Further extraction was given up, because the products in similar runs had been found of much less amount and, in addition, were mostly insoluble in glycol at the initial stage of subsequent fractionation.

3) This was very small.

4) The remaining unextracted gave biuret reaction intensively but Molisch reaction barely.

5) α-Naphthol-sulfuric acid test resulted as in the case of Fr. $A_{1b}$, namely this was revealed to still involve a glycidamin or a carbohydrate.

6) No precipitation occurred on further repetition of changing the pH.

7) From the filtrate and the supernatant liquid could be obtained alcohol-precipitable ubstances after condensation and after dialysis and condensation respectively, but they amounted very small and moreover gave almost no reaction with Molisch reagents.

8) Chargaff, Bancroft and Stanley-Brown, J. Biol. Chem., 1936, **115**, 149.

9) The original solution was prepared by taking up the substance in physiological saline followed by neutralizing with 5% $Na_2CO_3$.

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# Demonstration of a Neuroid Naevus (Masson).

By

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According to Masson (1926) and Ito (1946), all the pigment naevus should be a metamorphosed Schwannoma. The specimen, the subject of this paper, was taken at autopsy from a woman of 45 years, who died of chorion-epithelioma. There was in the anterior chest wall, a small pigment naevus, of which paraffin sections were made. The microscopic feature was of interest in showing that the '*neuroid bundles*' around a hair follicle continued to a neurofibromatous structure of the upper cutis smoothly, corresponding to the '*leaf-like laminae*.' The ordinary epithelioid appearance was rather limited to the superficial zone, where the naevus cells were filled with melanin pigment notably. There was no direct communication between the naevus cells and the epidermis, connective tissue fibers separating them everywhere. Clear cells (Merkel-Ranvier) were not numerous in the epidermis. The whole structure corresponds to a top-like formation (Takeuchi 1921) stereographically.

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# Brain Waves and the Localization of Cerebral Function.

By

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## Introduction.

The idea that the different areas of the cerebral cortex are concerned with different functions is so universally accepted that the anatomical evidence that quite different kinds of nerve cells are co-existing in one and the same area, hardly receives due attention. In the cortex, usually six layers are distinguishable, each of which consists of nerve cells of the same kind. If the functions of different kinds of nerve cells are to be different from one another, the function of the cortex should be different not so much from area to area as from layer to layer. This view which may be designated "laminar localization of cerebral function" against the areal localization, was advanced by brain-anatomists, Jakob,[1] Monakow[2] and others.

Jakob maintained that the outer layers of the cortex are sensory, while the inner are motory, and this view was supported by evidence that endings of corticopetal fibres are proved in the outer layers such as the lamina pyramidalis (III) and the lamina granularis interna (IV), and that the corticofugal fibres originate from the inner layers, i.e. the lamina ganglionaris (V) and the lamina multiforme (VI). Economo and Koskinas[3] summarize the inferences which can be deduced from morphological findings as follows: The function of the lamina zonalis (I) is chiefly associative, that of the L. gran. ext. (II) associative and commissural, that of the L. pyram. (III) sensory, associative and commissural, that of the L. gran. int. (IV) sensory and associative, and those of the L. gangl. (V) and the L. mult. (VI) motory.

This theory, though morphologically so well grounded, attracted little attention, especially among physiologists, because 1. there was formerly

no experimental means to study the function of each layer separately, 2. there are few clinical and pathological evidence supporting this view and 3. the latter seems to be contradictory to the doctrine of areal localization of cortical function. Whatever the histological ground of this theory may be, it will not generally be accepted without physiological supports. The discovery of brain waves has provided a new means of exploring the cerebral function and enables to investigate this question from a different angle. In the present communication these two theories will be criticized from the standpoint of electrophysiology.

## Results.

In animal, Kornmüller[4] stressed the differences in pattern of electroencephalograms (eeg) from cytoarchitecturally different areas in the rabbit, whereas Rheinberger and Jasper,[5] by permanent electrodes in the cortex of the unanesthetized cat, were unable to find consistent differences in different areas because of variations in the activity of one region at different times. In man, a number of investigators could find no definite difference in pattern among eeg taken from various regions of the head; although sometimes a localization of certain potential patterns is found in definite, decidedly large areas of the cortex, there is certainly not the precise localization of pattern that might be expected from the anatomical or physiological brain map.

It seems to me to be even unreasonable to expect a definite correlation between the pattern of eeg and the cytoarchitecture of the cortex, because the former is a dynamical aspect of the cortical function, whereas the latter is a statical feature of the cortex. If any property of the eeg is to be chosen for the comparison with the histological aspect of the cortex, a statistical measure of the eeg such as mean amplitude or mean period should be chosen because it can be regarded as statical feature of the cortical function.

### 1. Is there any Local Difference with regard to the Mean Period?

I and my colleague Tuziguti measured the mean period of eeg taken from various regions of the head to see whether any definite local difference could be found. Some data are shown in Table I. The local difference in the mean period remained in most cases within 5%; the greatest and the smallest value of the mean period from one and the same subject differed by only 0.001—0.005 sec. in 15 cases, 0.006—0.010 sec. in 10 cases and 0.011—0.014 sec. in 4 cases. Since differences of the same order were found also among the values obtained at one and the same region, but at different

TABLE I.

The mean period in sec. of eeg taken from various regions of the head in man. (Average of 400–1000 waves)

| Subject | Occipital | | Parietal | | Precentral | | Frontal | |
|---|---|---|---|---|---|---|---|---|
| | Left | Right | Left | Right | Left | Right | Left | Right |
| Komai | 0.112 | 0.108 | 0.111 | 0.110 | 0.115 | 0.112 | 0.115 | 0.111 |
| Tuziguti | 0.107 | 0.107 | 0.103 | 0.108 | 0.106 | 0.108 | 0.106 | 0.105 |
| Kikuti | 0.107 | 0.106 | 0.104 | 0.107 | 0.104 | 0.106 | 0.111 | 0.106 |
| Tamaru | 0.101 | 0.104 | 0.101 | 0.101 | 0.110 | 0.109 | 0.101 | 0.109 |
| Shozi | 0.103 | 0.099 | 0.100 | 0.104 | 0.102 | 0.100 | 0.104 | 0.098 |

times, the differences obtained above cannot be significant as local differences.

Jasper and Andrews[6] reported that eeg taken from the precentral region of man were richer in $\beta$-waves than those from other regions. If this were also the case in our subjects the mean period for the precentral region should be shorter than that for other regions, but no such tendency could be found in our experiment.

## 2. Regional Difference with regard to the Mean Amplitude.

While the attempt to find significant regional differences with regard to the mean period has failed, we have succeeded in discovering very distinct local differences with respect to the mean amplitude. As the details of the method and results of our experiment are reported elsewhere,[7][8] only the part necessary for the discussion of the present question will be mentioned in the following. Eeg were taken by monopolar leads with the ear lobe as reference point. To show the regionar difference clearly, the result was expressed as the distribution curve of the mean amplitude, an example of which is illustrated in Fig. 1 (A). The ordinates represent the mean amplitude, and the abscissae the position of the electrode on the median sagittal line from occiput to glabella. The curve (in full line) shows occipital, parietal and frontal elevations, the former two being separated by a deep dale which can always be demonstrated clearly if this region is explored in detail. The frontal elevation is usually less marked and may be divided into two subdivisions, i.e. precentral and frontal elevation. A similar distribution curve could be obtained whichever sagittal meridian might be taken. We have constructed such distribution curves in 27 cases, and confirmed that the distribution was little altered so long as the state of consciousness remained fairly constant. This shows that the distribution curve is a statical expression of the cortical function. There-

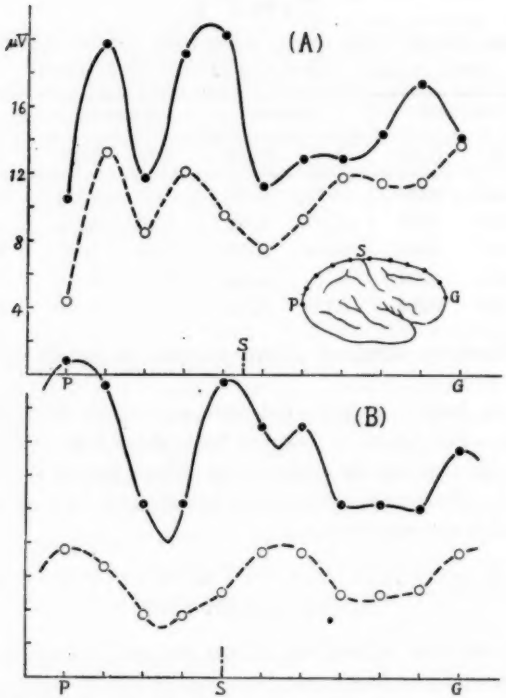


Fig. 1. (A) The distribution of the mean amplitude of α-waves over the cortex of man. Abscissæ: Distance from the protuberantia occipitalis externa along the median sagittal line of the head. One scale: 3 cm. Ordinates: Mean amplitude in μV. P: Protuberantia occip. ext., S: Sulcus centralis, G: Glabella. Full curve represents the distribution in the standard passive state of consciousness in the dark, and broken curve the distribution under visual stimulation by a constant illumination.

(B) Theoretical distribution curves. Full and broken curves correspond to those in (A) respectively. It was assumed that mean amplitude is proportional to the product of the number and the length of cortical cells. For the construction of the upper curve the whole cortical layers were taken into account, while the lower was constructed with only the data of the inner cortical layers V and VI.

fore, we can expect a definite relation between distribution curve and cytoarchitecture of the cortex.

It is beyond question that the potential at a point of the scalp is equal to the sum of the potentials caused by individual active cells, but it is not clear how the individual cells contribute to the sum of potentials. As to this point, the author advanced a theory which connects the mean amplitude $\bar{A}$ of α-waves with histological data such as the number of nerve cells and the length of the cells etc. The mean amplitude $\bar{A}$ is given by

the following formula, namely:

$$\bar{A}=\frac{\kappa\sqrt{\pi}\sum_i\sum_j\lambda_{ij}\sigma_{ij}\mu_{ij}\Omega_{ij}}{2\sqrt{N}}$$

in which $N$ is the number of all active cells in the cortex, $\kappa$ a physical constant concerning the electrical conductivity of the medium, $\lambda$ the correction term with respect to the orientation of nerve cells, $\sigma$ the number of active cells in volume element (unit area times thickness of the layer), $\mu$ the dipole moment of cells, $\Omega$ the solid angle subtended by a cytoarchitectonic area, and the symbols $\sum_i$ and $\sum_j$ mean summation with respect to areas and layers respectively.

The relation can be more simplified by taking into account that the potential at a point is determined chiefly by the area which lies directly beneath the exploring electrode, and that $\lambda$ equals nearly 1, as shown in my previous report.[10] Then the mean amplitude must be approximately proportional to the product of $\sigma$ and $\mu$ in that area. The number of active cells can be taken as proportional to the number of cells histologically determined, so that $\sigma$ is known, but the dipole moment $\mu$ of nerve cells is unknown at present. If it is, however, assumed that the asymmetry of potential at the cellular body is distributed along the longitudinal axis of nerve cells as is the case with muscular cells, the dipole moment must be proportional to the length of nerve cell bodies which is obtainable also from histological data of the cortex. Thus the mean amplitude at any region can be correlated to the histological data for that region.

Now the question arises which sort of cells in the cortex produces $\alpha$-waves. In the theory, the cells which are generating $\alpha$-waves are expressed by the term " active cells," but the theory itself cannot solve this question, so that it must be decided experimentally. In animal, this question could be solved, for instance, by thermocoagulation of cortical layers one by one or by picking up the potential of each layer by means of an insulated needle-electrode with an exposed tip inserted on to the layer in question, but it is difficult to apply these methods to normal subjects. The only way to choose is, therefore, to evaluate the product of the number and the length of cells for each layer of each cortical region from reliable histological data, and to see whether the distribution curve of the product so evaluated goes parallel to the distribution curve of the mean amplitude. In the present investigation the data of Economo and Koskinas[3] were used.

The distribution curve of the product so evaluated for any one of the cortical layers was found to be so dissimilar to the distribution curve for the mean amplitude that the production of $\alpha$-waves cannot be attributed to any one layer of the cortex. Both elevations, occipital and parietal, and the dale between these elevations could be imitated to a certain extent by

taking the sum of the products for the layers II, III and IV, but the precentral elevation could not be realized until the products for the layers V and VI were added to them. In short, the agreement was found to be satisfactory only when all the cortical layers were assumed to be participating in the production of $\alpha$-waves. The theoretical distribution curves are shown in Fig. 1 (B). The upper curve in this figure corresponds to the upper curve in (A), i.e. distribution curve of the measured mean amplitude. It will be seen from this figure that both curves agree with each other to the minute detail. If the number of cells were the only factor determining the mean amplitude, the occipital elevation would have to be highest of all, because the number of cells is greatest in this region as the result of special development of granular cells. As a matter of fact the occipital elevation cannot always be highest because of the small dimension of the granular cells. In the frontal part, the dimension of cell body is greater on an average, but the mean amplitude is smaller than in the posterior half of the head, and this is evidently due to the sparse distribution of cells in the frontal region. In the precentral region, the number of cells is so small that a dale instead of an elevation would be found in the distribution curve, if the number of cells were the sole determining factor; the small elevation found in the precentral region is to be attributed to the greater dimension of the cortical cells in this region such as pyramidal cells of Betz. At any rate, it must be emphasized that for the production of bioelectric potential the number of cells, their orientation in the tissue and the dimension of cell body should be taken into consideration.

### 3. The Effect of Sensory Stimulation upon the Distribution Curve of the Mean Amplitude.

To study the effect of visual stimulation upon the mean amplitude of $\alpha$-waves in various regions, eeg were taken under a constant illumination, the other conditions being held as the same as in the experiment mentioned above. The result is shown in broken line in Fig. 1 (A). As seen from this figure, the effect is more marked in the posterior half than in the anterior half of the head. Similar relations were obtained also in other cases, several of which are shown in Table II. It is to be noted that the effect is never limited to the occipital region in which the visual area is situated, but that the frontal regions which are remote from the visual area are more or less affected by visual stimulation. The decrease in $\alpha$-activity is not always most marked in the occipital region, and not always least in the forehead which is situated farthest away from the visual area. This fact cannot be explained by the assumption of electrical spread from the occipital region to the frontal. The effect must be transmitted through associative or commissural mechanisms from the visual area to other parts

TABLE II.

The effect of visual stimulation upon the mean amplitude $\bar{A}$ of $\alpha$-waves at various regions. O,P, Pr and F represent occipital, parietal, precentral and frontal regions respectively.

| Subject | Region | $\bar{A}$ in the dark | $\bar{A}$ under illumination | Difference |
|---|---|---|---|---|
| Tuziguti | O | 17.2 | 8.6 | 8.6 |
| | P | 20.1 | 9.5 | 10.6 |
| | Pr | 17.5 | 10.3 | 7.2 |
| | F | 17.6 | 9.1 | 8.5 |
| Hongo | O | 28.2 | 10.7 | 17.5 |
| | P | 30.6 | 8.6 | 22.0 |
| | Pr | 21.0 | 18.8 | 2.2 |
| | F | 16.4 | 8.2 | 8.2 |
| Miura | O | 15.1 | 6.3 | 8.8 |
| | P | 24.5 | 8.6 | 15.9 |
| | Pr | 23.7 | 11.9 | 11.8 |
| | F | 17.3 | 8.7 | 8.6 |
| Endo | O | 19.6 | 13.2 | 6.4 |
| | P | 19.2 | 12.0 | 7.2 |
| | Pr | 12.8 | 9.3 | 3.5 |
| | F | 14.2 | 11.3 | 2.9 |
| Sasaki | O | 11.8 | 3.0 | 8.8 |
| | P | 22.2 | 7.4 | 14.8 |
| | Pr | 20.1 | 11.3 | 8.8 |
| | F | 17.1 | 11.5 | 5.6 |
| Minegisi | O | 27.8 | 11.6 | 16.2 |
| | P | 32.6 | 16.9 | 15.7 |
| | Pr | 27.0 | 22.3 | 3.8 |
| | F | 22.– | 18.2 | 4.6 |

of the brain. As mentioned above, the outer layers of the cortex I, II, III and IV are said to be concerned with the receptive, associative and commissural functions while the inner layers V and VI belong to the effector mechanism.

Now the question arises whether the outer layers are chiefly involved in the process of perception in general. Our theory can predict what sort of change the distribution curve will undergo when the $\alpha$-waves of the outer layers are affected by sensory stimulation. Let the $\alpha$-waves of these layers be assumed, for simplicity, to be abolished completely by sensory stimulation, then only the $\alpha$-waves of the inner layers would remain, and the distribution curve would be such that drawn in broken line in Fig 1. (B). Under this assumption, the distribution curve would change from the upper curve into the lower in this figure. The effect of sensory stimulation is clearly more marked in the posterior half than in the anterior half of the head also in these theoretical curves. There exists some resem-

brance between the distribution curve of the measured mean amplitude in (A) and the theoretical in (B) (compare the two broken curves with each other). But there are surely some discrepancies between them, one of which is that the reduction of $\alpha$-waves is generally slighter in the experiment than in the theory, and the other of which is that the reduction in the frontal part is less than what the theory predicts. These disagreements, however, are not so essential, because they are certainly due to the unnatural assumption that the outer layers all over the cortex are to be affected equally and totally. In reality, the extent of areas and the number of cells which would be affected, must be different according to the kind and the strength of sensory stimulation, and these physiological mechanisms stand obviously beyond the scope of our theory.

## Discussion.

From the behavior of the $\alpha$-waves outlined above, it can be infered that the brain is acting as a whole, because its various parts are called into play simultaneously even when only one kind of receptor organ is stimulated. We can find no sign of the areal localization of cortical function so long as the activity of $\alpha$-waves is concerned. This seems to be inconsistent with the doctrine of cerebral localization. However, the $\alpha$-waves express nothing but one side of complicated electrical phenomena of the central nervous system.

According to Kornmüller[4] and others, the action potential or evoked potential takes place on sensory stimulation only within the limits of the sensory area the receptor of which is stimulated. This fact indicates that the cortex behaves according to the principle of areal localization, at least with respect to evoked potentials.

Now it is clear that neither of both theories of laminar and areal localization alone is sufficient even if the discussion is limited to the electrical phenomena. They describe different aspects of the cerebral function, and are never contradictory, but complementary to each other.

On the other hand, the present investigation gives an important suggestion as to the origine of the $\alpha$-waves in the cortex. Berger advanced a hypothesis that the three outer layers produce $\beta$-waves while the inner generate $\alpha$-waves. He believed that his hypothesis was based on the ingenious experiment of Dusser de Barenne and McCulloch,[11] who found that after the thermocoagulation of the three outer layers of monkey's cortex there remained only slow waves, rapid $\beta$-waves being entirely abolished. But they became thereafter aware of the deleterious effect of acids produced by the thermocoagulation, and performed an experiment anew, after the experimental animal had completely recovered from the damage of thermocoagulation. The result was quite different from that

of the previous experiment; both sorts of waves $\alpha$ and $\beta$ were found in a similar proportion to the eeg taken before the operation.

From this result it is probable that all layers are capable of producing both sorts of waves, but not yet conclusive enough. The fact that the mean amplitude of $\alpha$-waves is greatest in the occipital and parietal regions where the outer layers are well developed, and that the effect of visual stimulation is most marked also in these regions suggests an important rôle played by the outer layers in these respects. It is more important for this question that the agreement between experimental and theoretical distribution curves of the mean amplitude is never satisfactory unless all cortical layers are assumed to be participating in the production of $\alpha$-waves.

O'Leary and Bishop[12] reported that both sorts of waves $\alpha$ and $\beta$ could be obtained from all the layers of the visual area of rabbits by means of a needle-electrode. Marinesco, Sager and Kreindler[13] investigated eeg taken from the precentral region in three cases of amyotrophic lateral sclerosis and found distinct $\alpha$-waves of 9–11 Hz in all the cases. This can be regarded as another evidence that all the layers of the cortex are capable of producing $\alpha$-waves, because the layers III and V which occupy a greater part of the cortex in that region are said to be atrophic in this disease.

## Summary and Conclusion.

1. The mean amplitude of $\alpha$-waves in man shows characteristic local differences. On the contrary, the mean period of $\alpha$-waves shows no significant regional difference.

2. The mean amplitude is reduced by visual stimulation more or less all over the head, though in different proportions in different regions.

3. These phenomena were explained quantitatively on the basis of author's theory which relates the mean amplitude at a region to the histological data such as number of cortical cells, dimension of the cell body etc. at the region. Thus the relation between brain waves and the histological structure of the cortex has been established.

4. A satisfactory agreement between measured and theoretical mean amplitudes was attained only when the ability of producing $\alpha$-waves was attributed to all sorts of nerve cells in the cortex. From this, it may be concluded that all the cortical layers are participating in the production of $\alpha$-waves.

5. The effect of visual stimulation upon the mean amplitude could be interpreted as processes restricted chiefly to the outer layers of the cortex, and this offers an indirect support for the theory of laminar localization of cerebral function.

On the other hand, the behavior of evoked potentials which take place only within the limits of respective sensory areas is consistent with the

theory of areal localization. From the electrophysiological standpoint, these seemingly inconsistent theories are not contradictory, but complementary to each other.

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# Energy of Brain Waves and Energetics of the Brain.

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The electrical energy of brain waves originates from the chemical energy stored in nerve cells in the brain. It seems, therefore, to be possible to make use of brain waves for researches on brain metabolism, especially on such delicate changes of energy as could hardly be followed by usual biochemical methods. Judging from the behavior of brain waves, the amount of energy discharged as electrical energy must vary from moment to moment even under steady conditions of consciousness, for the amplitude of brain waves is always varying at random without any external causes.

Cencerning such fluctuations of amplitudes the author[1)2)] has established a statistical law which applies most satisfactorily to $\alpha$-waves of man. The law has the following analytical expression:

$$f(A)dA=2h^2Ae^{-h^2A^2}dA, \qquad (1)$$

where $f(A)dA$ represents the probability that the amplitude $A$ lies between $A$ and $A+dA$ and $h$ a constant.

The energy of waves is generally a function of amplitude and frequency. As the frequency of $\alpha$-waves is approximately constant, the energy $\varepsilon$ of $\alpha$-waves must be proportional to the square of amplitude $A$, namely: $\varepsilon=kA^2$. By making use of this relation and substituting the constant $h$ in (1) by another constant $\sqrt{k/\bar{\varepsilon}}$, we obtain the law of energy distribution for $\alpha$-waves as follows:

$$f(\varepsilon)d\varepsilon=\frac{1}{\bar{\varepsilon}}e^{-\frac{\varepsilon}{\bar{\varepsilon}}}\,d\varepsilon, \qquad (2)$$

where $f(\varepsilon)d\varepsilon$ represents the probability that energy $\varepsilon$ lies between $\varepsilon$ and $\varepsilon+d\varepsilon$, and $\bar{\varepsilon}$ the mean energy. Although this is nothing but another expression of the law of amplitude, it suggests us in what sort of form the distribution law of energy can be expressed. In the present communication, it will be investigated whether or not a distribution law of the same

form applies to any sorts of brain waves or a mixture of them.

## 1. Measurements of the Energy of Brain Waves.

The time average of energy dissipated during one period of waves is designated "power" in electric engineering. For a steady alternating current the power is constant and equal to the energy dissipated per sec., but when the amplitude varies from moment to moment like that of $\alpha$ waves, the power can be different from wave to wave. The power $P$ may be expressed as follows:

$$P=gV^2, \qquad (3)$$

where $V$ is the voltage amplitude, and $g$ is a factor dependent on the frequency. When the circuit consists of only resistances, $g$ equals one half the reciprocal of the total resistance, so that the power does not depend on the frequency. If the circuit contains a resistance $W$ and a capacity $C$ in series, then $g=\frac{W}{2(W^2+1/\omega^2C^2)}$, where $\omega$ is the circular frequency, and in this case the power evidently depends on the frequency. In general, living tissues show a capacitive action, which is especially so marked in the skin that the energy of electroencephalograms (eeg) taken from the scalp cannot be a function of the amplitude alone. How the factor $g$ depends on frequencies is not so simple in living tissues as in physical models, and demands special investigations.

TABLE I.

The factor g for measurement of the power of eeg in man. If the amplitude of brain waves is measured as the vertical distance from trough to crest of a wave, the values in this table must be divided by 8.

| Frequency (Hz) | g×21650 (Watt/volt²) | Frequency (Hz) | g×21650 (Watt/volt²) |
|---|---|---|---|
| 50 | 1.39 | 9.1 | 2.52 |
| 33.3 | 1.54 | 8.35 | 2.62 |
| 25 | 1.68 | 7.7 | 2.75 |
| 20 | 1.82 | 7.15 | 2.85 |
| 16.7 | 1.94 | 6.67 | 2.94 |
| 14.3 | 2.06 | 6.25 | 3.03 |
| 12.5 | 2.19 | 5.9 | 3.08 |
| 11.1 | 2.29 | 5.55 | 3.26 |
| 10 | 2.43 | 5 | 3.4 |

Motokawa and Iwama[3] measured the impedance of the head under the same conditions as in the electroencephalography and determined the factor $g$ for each sort of brain waves. The result is shown in Table I.

The theoretical ground for this procedure is given Appendix I of this paper. Table I shows that the factor $g$ can be regarded as fairly constant so long as the frequencies of brain waves are not so different from one another. It is a simple matter to evaluate the energy for a single sort of waves, but a special consideration is necessary for treatment of a mixture of different kinds of waves. Attention must be paid first of all to the main rhythm or predominant waves when a second sort of waves is superimposed upon them. The power is evaluated for the main rhythm alone, and then it is corrected with respect to the energy of the superimposed waves, if this cannot be neglected against the energy of the main rhythm.

In carrying out the correction, the mean of the energy of each superimposed wave, but not the sum of them should be added to the power of the basic wave. In such portions of the eeg where no superposition of waves is found, the power of every wave must be evaluated independently, how minute its amplitude may be. It is a matter of consideration whether or not very slow deflections of base-line should be taken into account. If they evidently originate from nerve cells in the brain, their energy should, of course, be appreciated, but in reality, it is no easy matter to decide the origine of this sort of electrical change.

## 2. The Exponential Law of Energy Distribution in the Electroencephalogram.

The distribution of energy was investigated on eeg of normal subjects. In one series of experiment the energy was taken as a quantity proportional to the square of amplitude, and in another the corrections were made with regard to the factor $g$ and to the energy of superimposed waves, in order to get idea of the order of correction. Energy was measured for one wave after another over the whole length of an eeg including several hundred waves, and classified into several groups. To see whether the exponential law mentioned above held or not, the logarithms of the number of appearance for each energy group were plotted as ordinates against the amount of energy as abscissae. So long as eeg of normal subjects are concerned, a linear relationship was always proved in both series of experiment (Fig. 1). This is because the frequency of the main rhythm, i.e. $\alpha$-waves is approximately constant, and the amplitude of $\beta$-waves superimposed upon the main rhythm is small enough against that of the $\alpha$-waves.

Eeg of some epileptic patients were also investigated. They were composed of a variety of waves such as $\alpha$, $\beta$ and $\delta$, nevertheless the relation was linear, indicating that the exponential law holds. In this case the corrected data with respect to the factor $g$ and to the energy of superimposed waves showed a better fitness. To test the validity of the exponential law in animal, eeg of rabbits were investigated. They were much more ir-

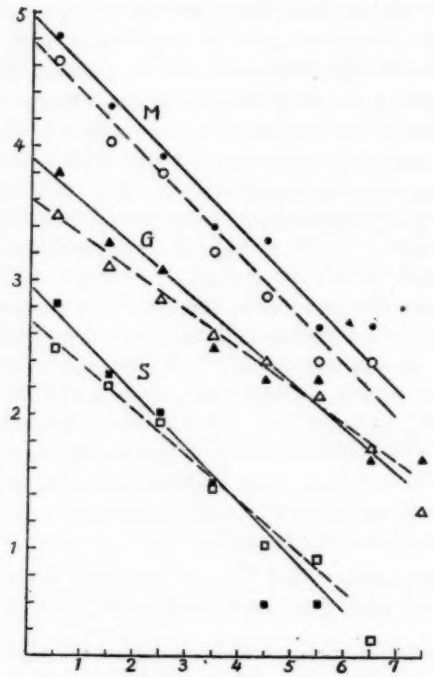


Fig. 1. Experimental verification of the exponential law of energy distribution in eeg. Ordinates: Logarithms of the frequency of appearance. Abscissæ: Energy per unit time (power). For the experimental series expressed by solid marks the energy was assumed to be proportional to the square of amplitude, and for the one expressed by empty marks the energy was correctly measured. M, G and S are the results for different individuals. Ordinates for the data of different cases are plotted so as to be shifted to one another by one logarithmic scale, and ordinates for two different series of the same individual are shifted to each other by 0.2 log. scales.

regular than those of man, none the less the exponential law held. Deflections of base-line which were marked in some cases, were not taken into account, because their cerebral origin was not certain and their appearance in eeg was not so frequent as sufficient for a statistical treatment.

## 3. A Statistical Theory of the Energy Distribution in the Electroencephalogram.

The exponential law applies to all sorts of eeg available so that it can be regarded as a general law for brain waves. Masuyama[4] deduced this law from the following assumptions: 1. The total sum of energy which can be converted into the energy of brain waves, is constant. 2. In the

brain there is a great number of energy groups, any one of which comes to be measured as electrical energy. 3. The amount of energy contained in each group can take any value between the constant sum of energy and zero. The formula at which he arrived after some mathematical treatment is as follows:*

$$f(\varepsilon)d\varepsilon=\frac{n-1}{G}\left(1-\frac{\varepsilon}{G}\right)^{n-2}d\varepsilon, \qquad (4)$$

where $f(\varepsilon)d\varepsilon$ represents the probability that energy $\varepsilon$ lies between $\varepsilon$ and $\varepsilon+d\varepsilon$, $n$ the number of energy groups, and $G$ the sum of energy in the brain. By putting

$$\bar{\varepsilon}=G/n \qquad (5)$$

and $n\to\infty$, the relation (4) reduces to the exponential formula (2), where $\bar{\varepsilon}$ expresses the mean energy. When $n$ is greater than 10, the exponential formula can be employed instead of the formula (4) within the limits of experimental error, as pointed out by Masuyama.

It seems to me, however, that the first assumption of this theory is too rigid for processes in a living system, the brain, in which the amount of free energy cannot be assumed as constant without any ground. I have become aware that the same conclusion can be reached without resort to this assumption. Instead of this, it is only necessary to assume that the sum of free energy cannot exceed a certain upper limit, and this is almost selfevident so long as a biological system is concerned. Through some mathematical treatments which are reproduced in Appendix II of this paper, I reached the following formula:

$$f(\varepsilon)d\varepsilon=\frac{n}{G}\left(1-\frac{\varepsilon}{G}\right)^{n-1}d\varepsilon, \qquad (6)$$

where $G$ is the upper limit of the sum of energy, and other notations are the same as in (4). This formula is also equivalent to the exponential formula (2) for greater values of $n$ than about 10. The number of groups $n$ is quite arbitrary, provided that it is greater than about 10, and the sum of energy can vary between $G$ and 0. It is to be noted that the exponential law holds under such general conditions. However, the assumption that the sum of energy can vary between $G$ and 0, does not always mean that every value between these limits is equally probable. There is a number of physiological facts suggesting that the free energy of the brain remains stationary within certain narrow limits. To see whether our theory conforms to the physiological evidence, I calculated from the same theoretical basis as above the probability $\varphi(S)dS$ that the sum of energy $S$ lies between $S$ and $S+dS$, and obtained the following relation:

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* In Masuyama's original $n$ and $n-1$ are written in place of $n-1$ and $n-2$ in (4) respectively. This is due to error of his calculation.

$$\varphi(S)dS=\frac{n}{G^n}S^{n-1}dS. \qquad (7)$$

This implies that the sum of energy lies probably very near the upper limit $G$. For example, the probability that the sum of energy remains over 80 per cent of $G$, is greater than 0.9, as can be proved by the following calculation:

$$\int_{0.8G}^{G}\varphi(S)dS=\left[\left(\frac{S}{G}\right)^n\right]_{0.8G}^{G}=1-0.8^n>0.9 \qquad (8)$$

when $n$ is greater than 10. The sum of energy, therefore, is practically constant and approximately equal to the maximum. The constancy of the sum of energy was the assumption of Masuyama's theory, but in my theory, it is a conclusion instead of an assumption.

Important as its conclusion is, our theory is surely so abstract that it is difficult to interpret the basic assumptions of this theory in appropriate physiological terms. The author[1)5)] put forward another theory to account for the law of amplitude. The assumption of this theory was that electrical pulsations of individual cells or cell groups were completely out of phase, though their periods were equal to one another. Such a chaotic phase relation would be realized in such a way that nerve cells or cell groups take part in the production of brain waves freely at any moment and arbitrarily cease their activity at another moment so that the number of cells active must be a fraction of the whole number of nerve cells. The same mode of activity could be concluded from the assumption of the present theory that any one of the whole groups of energy comes to our observation as electrical energy. This mode of action seems to be most suitable for organs which are obliged to work incessantly.

## 4. Energetics of the Brain.

The gaseous metabolism of the body increases but little by mental activity, as pointed out by Rubner, Benedict, Cramer and others, but this does not mean that the brain consumes only little oxygen. On the contrary, the oxygen consumption of the brain is so active that the amount of oxygen consumed per gr. per hour is said to be about twenty times as much as that of resting muscle. The little increase in gaseous metabolism by mental activity is, therefore, to be so interpreted that the difference between two mental states, active and inactive cannot be great because the metabolism in the inactive state is already close to its maximum.

The electrical behavior of the brain is consistent with this metabolic relation. The energy of $\alpha$-waves which fully develop in the passive state of consciousness, is no less than that of action potentials appearing in the active state. No cessation of electrical activity occurs even in sleep and

narcosis. Far from it, a marked increase of electrical energy can be observed at some stage of sleep and narcosis, as will be explained below.

Our theory outlined above claims that the sum of energy is held at the maximum even in the passive state of consciousness, and this seems to receive experimental support from the researches on metabolism. The maintenance of the maximum brain energy is a natural outcome of the statistical mode of action of nerve cells, which is exemplified above all in the law of energy distribution of brain waves.

The brain is one of the organs most susceptible to deprivation of oxygen, and this fact is often explained as follows: The brain is a highly differentiated organ lying at the latest stage of evolution, so that it is most sensitive to oxygen defficiency. This way of explanation is so biological and very easy to understand, but hardly elucidates the mechanism which underlies so high susceptibility to oxygen deficiency.

I propose as a possible explanation the following: The slightest deprivation of oxygen would cause the utilizable energy of the brain to decrease and consequently to cease to be maximum, and this means that the function of the brain ceases to be normal, because the maintenance of the maximum energy itself is the mode of normal function of the brain.

To guarantee our brain against oxygen deficiency, our body is provided with a number of nervous mechanisms regulating blood supply to the brain; for example, when we are exposed to oxygen deficiency the reflex mechanisms make the heart accerelate its function, the lung ventilate air more vigorously, peripheral vessels contract, the coronal and cerebral arteries, however, dilate to supply more blood to the brain. When oxygen supply, in spite of these compensating actions, falls short of consumption, the utilizable energy of the brain inevitably decreases. In terms of our theory, such a situation implies a decrease of the probability that the sum of energy lies near the maximum. As the theoretical relations (7) and (8) indicate, a decrease of this probability is realized only by a decrease in the number of groups $n$. A decrease of $n$ means in turn an increase of the mean energy according to the relation (5).

Our theory, therefore, predicts that oxygen deficiency must cause an increase in the mean energy of brain waves. This inference may seem to be somewhat surprising, for one might expect that the energy of brain waves would decrease, but never increase as the result of oxygen deficiency. In reality, however, $\delta$-waves with great amplitudes appear and cause the mean energy of brain waves to increase, whenever the functional state of the brain is abnormal. To study this relation more quantitatively, I and my colleague Iwama measured the energy of brain waves of normal subjects before, during and after the inhalation of a gas mixture of nitrogen and air. The result is shown in Table II. It indicates that the effect of oxygen

TABLE II.

The effect of oxygen deficiency upon the mean energy of brain waves in man.

| Subject: Takizawa $O_2$-content: 7.4% | | Subject: Watanabe $O_2$-content: 6% | | Subject: Motokawa $O_2$-content: 7.64% | |
|---|---|---|---|---|---|
| | Mean energy | | Mean energy | | Mean energy |
| Before (normal air) | 946 | Before | 115 | Before | —— |
| 1 minute inhalation | 650 | 1 minute 〃 | 141 | 1 min. 〃 | 94 |
| 3 minutes inhalation | 1160 | 3 min. 〃 | 409 | 2 min. 〃 | 118 |
| 5 minutes inhalation | 1771 | 6 min. 〃 | 498 | 4 min. 〃 | 168 |
| 2 minutes after return to norm. air | 680 | 2 min. after return to norm. air | 93 | 1 min. after ret. to norm. | 98 |
| 3 minutes after return to norm. air | 873 | 3 min. 〃 | 116 | 2 min. 〃 | 103 |

deficiency is very striking and reversible; an increase of about 100 per cent in the mean energy was brought about by inhalation of four to six minutes in all cases examined. Similar results have been reported by several investigators such as Berger,[6] Gibbs and Davis,[7] Lennox, Gibbs and Gibbs[8] and others, but have never been dealt with from the energetic point of view.

Some authors advanced the hypothesis that the increase of amplitude found under various abnormal conditions is due to increased synchronization of electric pulsations from individual nerve cells. This may be admitted as one of possible mechanical interpretations of this phenomenon, but it is not clear why synchronization is increased under such conditions. In our theory, it is clearly shown that a decrease of free energy is the direct cause for a decrease in the number of groups $n$ which is inevitably followed by an increase in the mean energy, because the latter varies inversely as the number of groups. At any rate, it deserves attention that various phenomena which do not seem to be closely related to one another, i.e. the maximum energy of the brain, the exponential energy distribution of brain waves, the increase in the mean energy due to oxygen deficiency etc. can be accounted for from one and the same theoretical ground.

## Appendix.

### I. Theoretical Ground for Energy Measurement of Brain Waves.

In the following calculation of the power $P$ of brain waves, the equivalent impedance model (C) in Fig. 2 of our previous work[3] was used. Let $T$ be the period $(=\frac{2\pi}{\omega})$ and $\theta$ be the phasedifference between voltage and current, then by definition of the power the following relation is obtained:

$$P=\frac{1}{T}\int_0^T I_0 \sin \omega t.\ V_0 \sin(\omega t+\theta)\ dt=\frac{1}{2}I_0V_0 \cos\theta, \quad (9)$$

where $I_0$ and $V_0$ are the current and voltage of brain waves respectively. In the model referred to above the following relations exist:

$$I_0=V_0/\sqrt{(R+r)^2+1/\omega^2C^2}, \quad (10)$$

$$V=rV_0/\sqrt{(R+r)^2+1/\omega^2C^2}, \quad (11)$$

$$\cos\theta=(R+r)/\sqrt{(R+r)^2+1/\omega^2C^2}, \quad (12)$$

where $V$ is voltage of brain waves measurable from outside, $R$ a resistance dependent on the frequency, $C$ capacity coupled with $R$ in series, and also dependent on the frequency, and $r$ a constant shunt-resistance for $R$ and $C$. If $I_0$, $V_0$ and $\cos\theta$ in (9) are expressed by the measurable quantities $R$, $r$ and $V$ according to (10), (11) and (12), the factor $g$ mentioned above is determined as follows:

$$P=gV^2, \qquad g=\frac{1+R/r}{2r}. \quad (13)$$

As to the real measurement of $R$ and $r$ our paper cited above must be consulted.

### II. Deduction of the Exponential Law of Energy Distribution.

If $G$ represents the upper limit of the sum $S$ of energy, and $\varepsilon_i$ is energy of the $i$-th group, our assumptions can be expressed analytically as follows:

$$G \geqq S \equiv \varepsilon_1+\varepsilon_2+\cdots\cdots+\varepsilon_n \geqq 0, \quad (14)$$

$$G \geqq \varepsilon_i \geqq 0 \quad (15)$$

A energy state which satisfies (14) and (15) can be expressed geometrically by a point within a $n$-dimensional polyhedron vertices of which lie at $(0,0,\cdots\cdots 0)$, $(G,0,0\cdots\cdots 0)$, $(0,G,0,\cdots\cdots 0)\cdots\cdots(0,0,0,\cdots\cdots G)$ respectively. If it is assumed that the probability density is homogeneous within this polyhedron, the probability that $\varepsilon_i$ lies between $\varepsilon_i$ and $\varepsilon_i+d\varepsilon_i$, must be proportional to the volume element of the polyhedron limited by $\varepsilon_i=\varepsilon_i$ and $\varepsilon_i=\varepsilon_i+d\varepsilon_i$. If $\rho$ is the proportionality factor,

$$f(\varepsilon_i)d\varepsilon_i=\rho d\varepsilon_i\int_0^{G-\varepsilon_i}\int_0^{G-\varepsilon_i-\varepsilon_1}\cdots\cdots\int_0^{G-\varepsilon_i-\varepsilon_1\cdots\cdots-\varepsilon_{n-1}} d\varepsilon_n\, d\varepsilon_{n-1}\cdots\cdots d\varepsilon_1$$

$$=\frac{\rho\,(G-\varepsilon_i)^{n-1}}{(n-1)!}d\varepsilon_i.$$

The factor $\rho$ is determined by the condition of probability $\int_0^G f(\varepsilon_i)d\varepsilon_i=1$, and we obtain

$$f(\varepsilon)d\varepsilon=\frac{n}{G}(1-\frac{\varepsilon}{G})^{n-1}d\varepsilon, \tag{16}$$

where the suffix $i$ was omitted because the same relation holds for any energy group. Since our theory assumes that the energy of any one group is converted into that of brain waves, the relation (16) represents the distribution law of energy of brain waves. Put $\bar{\varepsilon}=G/n$, and $n\to\infty$, then this equation reduces to the exponential formula (2).

Now we must calculate the probability for the sum of energy $S$ on the same basis. The equation $S=\varepsilon_1+\varepsilon_2+\cdots\cdots+\varepsilon_n$ is a $n$-dimensional hyperplane, if $S$ is assumed to be constant. The probability that $S$ lies between $S$ and $S+dS$ must be proportional to the volume element of the polyhedron limited by the hyperplanes $S=\varepsilon_1+\varepsilon_2+\cdots\cdots+\varepsilon_n$ and $S+dS=\varepsilon_1+\varepsilon_2+\cdots\cdots+\varepsilon_n$, and this volume is equal to $\frac{\sqrt{n}}{(n-1)!}S^{n-1}dS$. If $\mu$ is the proportionality factor, the probability

$$\varphi(S)dS=\frac{\mu\sqrt{n}}{(n-1)!}S^{n-1}dS.$$

If the factor $\mu$ is determined by the condition $\int_0^G\varphi(S)dS=1$, then the equation (7) in the text

$$\varphi(S)dS=\frac{n}{G^n}S^{n-1}dS$$

is obtained.

## Summary.

Electrical energy per unit time of brain waves was measured on electroencephalograms of man and animal, and the following law was established with regard to the distribution of energy in the electroencephalogram, namely:

$$f(\varepsilon)\,d\varepsilon=\frac{1}{\bar{\varepsilon}}e^{-\frac{\varepsilon}{\bar{\varepsilon}}}d\varepsilon,$$

where $f(\varepsilon)d\varepsilon$ represents the probability that energy $\varepsilon$ lies between $\varepsilon$ and $\varepsilon+d\varepsilon$, and $\bar{\varepsilon}$ the mean energy. To deduce this law theoretically from plausible assumptions, a statistical theory was advanced. An important conclusion from this theory is that the utilizable energy of the brain must be kept constant at its maximum, and this inference seems to be consistent with experimental evidence on brain metabolism. It can be explained

also from this theory that the mean energy of brain waves increases under unfavorable conditions such as oxygen deficiency, narcosis etc.

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# Biochemical Studies on Carbohydrates.

## CXI. On Snail Enzymes.

Second Report: Mannosidases.

By

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α-Mannosidase was first discovered in 1921 by Hérissey[1] in legume seeds and later on exhaustive investigations were accomplished by Helferich and co-workers[2] regarding the ones in almond and alfalfa seeds. However, of β-mannosidase and, in animal tissues, also of α-mannosidase we have never before been informed. I found out these enzymes in the snail liver extract, though not equally active. α-Phenol- and α-methyl-mannoside were cleft at the maximum at pH's 4.1 and 4.5 respectively and β-phenolmannoside at pH 4.5, namely, the optimum pH's of the α- and β-mannosidase resembled each other. On the contrary, however, the resistance of the enzymes to heat showed remarkable divergency which enables one to gain a solution containing α-mannosidase alone. The details concerned will be seen in the experimental part. Tissues of higher animals appear to be devoid of these enzymes.

## Experimental.

### Enzyme Solutions.

#### *I. The snail enzyme solution.*

It was prepared in a like manner as communicated in the first report[3] taking the same stock porridge. But, this time, to dissolve the half dried alcoholic enzyme precipitate, the aicidified water was used as much as 3/2 the volume instead of double the volume of the clarified stock solution and the extract obtained was further decolorized by treatment with purified[4] " charcoal, animal " E. Merck (0.1 gm. per cc.). The solution, which was still tinged yellow, was kept for the use at 0°C under saturation with toluene. It gave Molisch reaction but was almost biuret-negative.

Throughout the whole course of the present investigation one and the same enzyme solution was employed.

### *II. Canine pancreatic juice and the extracts of bull liver and rabbit liver, pancreas and kidney.*

The pancreatic juice collected from a dog with a fistula was evaporated to dryness under reduced pressure and preserved. For the examination it was dissolved in water in the proportion of 32 mgm. in 2 cc., and the centrifuged clear supernatant liquid, either as such[5] or after aicidfying to pH 4.5[6] (with 2 drops of 1% acetic acid), was adapted to incubation with the substrates. The organs were minced by means of a masticator, mixed with an equal weight of water and after addition of 12.5 cc. (in the case of ox liver) or 1.25 cc. (in the cases of rabbit organs) of toluene for 100 gm. tissue, maintained at 37°C for 48 hours with occasional shaking. After acidification and centrifugation, the dark brown supernatant fluids were precipitated by the use of alcohol and subsequent operations were carried out as when the snail enzyme solution was prepared. Decolorization with charcoal was omitted, when the extent of the enzymic digestion was intended to estimate by the iodometric titration alone.

#### Substrates.

$\alpha$- and $\beta$-phenolmannoside (Helferich and Winkler[7]) and $\alpha$-methylmannoside (E. Fischer[8]) were employed. Under the conditions, which will be described below, the substrates rotate D-light:

| | $\alpha$ (degree) | |
|---|---|---|
| | Before scission | When totally hydrolyzed |
| $\alpha$-Phenol-d-mannoside ($[\alpha]_D = +113.5°$) | +1.22 | +0.11 |
| $\alpha$-Methyl-d-mannoside ($[\alpha]_D = +79.2°$) | +0.61 | +0.10 |
| $\beta$-Phenol-d-mannoside ($[\alpha]_D = -71.6°$) | −0.77 | +0.11 |

#### Methods.

Substrate-buffer solutions were prepared by dissolving 0.054 mol of phenolmannosides or 0.051 mol of $\alpha$-methylmannoside in 1 cc. of a buffer solution (pH$\leqq$2.9: Sörensen's 0.1 n. citrate solution, pH 3.2–6.2: Michaelis' 0.1 n. acetate solution, pH$\geqq$7.0: Michaelis' 0.1 n. phosphate solution) saturated with toluene. 1 cc. of them and 0.3 cc. of an enzyme solution were mixed and incubated in a water thermostat at 37°C. When

the digestion was optically traced, the mixtures were alkalized by addition of 10 mgm. of potossium carbonate and filled up in a 1 dm. long tube, and for iodometry (Macleod and Robison[9]) they were cooled in ice and 0.5 cc. was taken out.

In the control runs the substrate-buffer solutions were replaced with the buffer solutions containing no substrate.

## Results.

### *I. On the snail enzymes.*

The constant of reaction velocity was calculated out by the formula $k=\frac{1}{t_{min}}\times 1n\ \frac{100}{100-\%\text{cleavage}}$. In the controls the optical rotatory power (−0.01°) showed no change.

1. Optimum pH's for the action of the mannosidases are seen in Table I.

TABLE I.

Optimum pH's of the Snail Mannosidases.

$\alpha_1$: initial rotation. $\alpha_2$: rotation after incubation.

| Substr. / pH | α-Phenol-d-mannoside (Incub.: 10 min.) | | | α-Methyl-d-mannoside (Incub.: 60 min.) | | | β-Phenol-d-mannoside (Incub.: 10 min.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ |
| 3.2 | +0.10 | 9.0 | 9.42 | +0.01 | 2.0 | 0.30 | | | |
| 3.8 | +0.13 | 11.7 | 12.43 | +0.07 | 13.7 | 2.35 | −0.02 | 2.3 | 2.33 |
| 4.1 | +0.15 | 13.5 | **14.49** | +0.09 | 17.6 | 3.32 | −0.03 | 3.4 | 3.45 |
| 4.5 | +0.10 | 9.0 | 9.42 | +0.14 | 27.5 | **5.35** | −0.04 | 4.5 | **4.60** |
| 5.0 | +0.06 | 5.4 | 5.55 | +0.11 | 21.6 | 4.05 | −0.01 | 1.1 | 1.15 |
| 5.8 | +0.05 | 4.5 | 4.60 | | | | | | |
| 6.2 | | | | +0.07 | 13.7 | 2.35 | | | |

2. The hydrolysis velocity diminished with the progress of digestion, suggesting the occurrence of the reverse reaction. Table II shows the courses of cleavage at the optimum pH's.

3. Inactivation by heat. Parts of the enzyme solution were heated in stoppered tubes for 30 minutes at various temperatures and after cooling, 0.3 cc. was pipetted out from each for determining the residual activity. Opalescence appeared, when heated at 60°C. At higher temperatures flocculi precipitated, but the precipitates were subjected to the use together with the mother liquid after shaking to equal dispersion. (Table III).

TABLE II.

Hydrolysis Velocity of Mannosides by the Snail Enzymes.

| Substr. / Time of incub. min. | α-Phenol-d-mannoside (Digest. at opt. pH, 4.1) | | | α-Methyl-d-mannoside (Digest. at opt. pH, 4.5) | | | β-Phenol-d-mannoside (Digest. at opt. pH, 4.5) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ |
| 5 | +0.08 | 7.2 | 14.93 | | | | | | |
| 10 | +0.15 | 13.5 | 14.49 | | | | −0.04 | 4.5 | 4.60 |
| 15 | | | | +0.06 | 11.8 | 8.38 | | | |
| 20 | +0.21 | 18.9 | 10.46 | | | | −0.07 | 8.0 | 4.16 |
| 30 | | | | +0.10 | 19.6 | 7.26 | | | |
| 40 | +0.32 | 28.8 | 8.48 | | | | −0.10 | 11.4 | 3.02 |
| 60 | +0.45 | 40.5 | 8.64 | +0.14 | 27.5 | 5.35 | | | |
| 90 | | | | +0.16 | 31.4 | 4.18 | | | |

TABLE III.

Heat Inactivation of the Snail Mannosidases.

Incubated for 10 minutes at the opt. pH's (4.1 and 4.5).

| Substr. / Temp. of heating °C | α-Phenol-d-mannoside | | | β-Phenol-d-mannoside | | |
|---|---|---|---|---|---|---|
| | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ | $\alpha_1-\alpha_2$ | Cleavage % | $k\times10^3$ |
| 50 | +0.14 | 12.6 | 13.45 | | | |
| 60 | +0.13 | 11.7 | 12.43 | −0.02 | 2.3 | 2.33 |
| 70 | +0.10 | 9.0 | 9.42 | 0.0 | 0.0 | 0.0 |
| 80 | 0.0 | 0.0 | 0.0 | | | |

β-Mannosidase was destroyed at 70°C entirely, whereas more than half of the α-enzyme still remained active at this temperature.

4. The activity of α-mannosidase was found not to decrease within a month of preservation at 0°C. For the measurement α-phenolmannoside was employed. pH of the incubation mixtures was 4.1 and the estimation was made every two days at first and afterwards every three days, but always $k\times10^3$'s of 14.93 and of 14.49 were given respectively in 5 and 10 minutes' incubation.

*II. On the extracts of mammal organs and pancreatic juice.*

The cleavage of α-phenolmannoside was examined at pH 4.5 in a like manner as above. The time of incubation was 60 minutes for bull liver,

60 and 120 minutes for the pancreatic juice and 30 minutes for rabbit organs. Measured optically as well as iodometrically for bull liver, optically for the pancreatic juice[10] and iodometrically for rabbit organs. In any case neither the optical power nor the iodine use underwent alteration.

Many thanks are due to the Grant Committee for Scientific Researches of the Education Department for a grant which covered the costs of this investigation. H. Masamune.

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5) Precipitation occurred when the liquid was mixed with the substrate-buffer solution.

6) When acidified, the solution became turbid, but it was adapted to incubation immediately as such.

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10) Optical measurement was made after centrifuging off of the precipitates.

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# Biochemical Studies on Carbohydrates.

## CXII. On Snail Enzymes.

Third Report: Galactosidases.

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With respect to $\alpha$- and $\beta$-galactosidase several physical properties were examined to devise a method of separating them from each other. Heating and dialysis were of no use. Optimum pH's for their action were also found not so markedly divergent, but still the purpose was attained by adsorbing the $\alpha$-enzyme to kaolin.

## Experimental.

### Enzyme Solutions.

Prepared as in the second work.[1] But here the solutions obtained by dissolution of the alcoholic enzyme precipitates and non-decolorized were applied—"original" solutions.

### Substrates.

For the aim in view, $\alpha$- and $\beta$-methylgalactoside and $\alpha$-phenolgalactoside were prepared according to Robertson & Lamb,[2] to Müller[3] and to Helferich and Appel[4]. And, besides, commercial lactose P. J. was taken as a substrate.

### Methods.

The activity of the present enzymes was measured in a manner adapted to the mannosidases.[1] Generally the amounts as follow were dissolved in 1 cc. of the buffer solutions: methylgalactosides, 0.047 mol; $\alpha$-phenolgalactoside, 0.054 mol; lactose, 0.053 mol. However, in case diluted enzyme solutions were examined, substrate and buffer solutions of higher concentrations were used so as to prepare incubation mixtures of

TABLE

Optimum pH's for

Incubation: 30

| Enz. sol. / Substr. / pH of incub. | Original solution | | | | | | Treated with | | |
|---|---|---|---|---|---|---|---|---|---|
| | α-Phenolgalactoside | | | Lactose | | | α-Phenolgalactoside | | |
| | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ |
| 1.5 | | | | 1.78 | 20.7 | 7.7 | | | |
| 2.2 | 1.07 | 0.1 | 0.03 | 1.85 | 21.6 | 8.1 | 0.94 | 1.1 | 0.4 |
| 2.5 | | | | 1.91 | 22.3 | **8.4** | | | |
| 2.9 | | | | | | | | | |
| 3.2 | 9.63 | 11.4 | 4.0 | 1.64 | 19.2 | 7.1 | 8.45 | 10.0 | 3.5 |
| 3.8 | 9.84 | 11.6 | **4.1** | | | | 8.56 | 10.2 | **3.6** |
| 4.0 | | | | | | | | | |
| 4.1 | | | | | | | | | |
| 4.5 | 8.46 | 10.0 | 3.5 | 1.46 | 17.0 | 6.1 | 8.51 | 10.1 | **3.6** |
| 5.0 | 5.39 | 6.4 | 2.2 | | | | 7.61 | 9.0 | 3.1 |
| 5.8 | 1.25 | 0.2 | 0.07 | 1.14 | 13.8 | 4.9 | 4.83 | 5.7 | 1.9 |

* 0.005 n. solution.

2 hours and filtered.

tap water. The volume of the solution increased from 10 cc. to 12 cc. so that 0.36 cc. of it and

similar composition as the usual. Extents of digestion were always calculated from iodometric titration.

## Results.

$k$ in the appended tables signifies the velocity constants of hydrolysis expressed as a unimolecular reaction.

*Exp.* 1. *Optimum pH's.* Their sites appeared to depend extremely upon impurities in the enzyme solutions, which was more the case in the $\beta$-enzyme than in the $\alpha$, and even among the original solutions, the enzymes did not always exhibit the maximum activity at one and the same pH each (the optimum pH's varied from 3.2 to 3.8 in $\alpha$-galactosidase and from 2.5 to 3.5 in the $\beta$). As is illustrated in Table I, treatment with charcoal and dialysis shifted the optimum pH's toward the less acid side, and, similarly as preservation did (Table II), decreased the activity especially that of $\beta$-galactosidase.

*Exp.* 2. *Effect of heat.* Parts of an original solution, whose optimum pH's for the $\alpha$- and $\beta$-enzyme were 3.8 and 3.2, were heated with an equal volume of the buffer solutions of pH 4.5 and 3.2 (in stoppered tubes). The duration of heating was 30 minutes. When cold, the activity of the enzymes was measured at the optimum pH's mentioned. The pH-adjustment was

I.

Snail Galactosidases.

minutes at 37°C.

| charcoal† | | | After dialysis‡ | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lactose | | | α-Phenolgalactoside | | | Lactose | | |
| Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ |
| 0.88 | 10.3 | 3.6 | 3.82 | 4.5 | 1.5 | 0.88 | 10.2 | 3.7 |
| 1.03 | 12.0 | **4.3** | | | | | | |
| 1.03 | 12.0 | **4.3** | 8.28 | 9.8 | 3.4 | 0.95 | 11.1 | 3.9 |
| | | | 8.30 | 9.9 | **3.5** | 0.97 | 11.4 | 4.1 |
| 1.00 | 11.7 | 4.1 | | | | | | |
| | | | 8.52 | 10.2 | **3.6** | 0.98 | 11.4 | 4.1 |
| 0.88 | 10.3 | 3.6 | 7.80 | 9.2 | 3.2 | 1.04 | 12.2 | 4.4 |
| | | | 6.20 | 7.3 | 2.5 | 1.23 | 14.4 | **5.3** |
| 0.60 | 7.0 | 2.4 | 3.70 | 4.4 | 1.5 | 0.42 | 4.8 | 1.6 |

† The original solution was shaken with purified[8] animal charcoal (0.1 gm. per cc.) for

‡ Dialysis through an intestine membrane was continued for three days against running

0.64 cc. of 1.2 times concentrated buffer-substrate solutions were mixed for the incubation.

## Table II.

### Activity of the Galactosidases at Various pH's after Preservation.

The original solution in Table I was preserved for 2 weeks at 5–10°C and the enzymic activity was measured after centrifuging off of some precipitate which appeared. Incubation: 30 minutes at 37°C.

| Substr. / pH of incubation | α-Phenolgalactoside | | | Lactose | | |
|---|---|---|---|---|---|---|
| | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ |
| 1.5 | | | | 0.66 | 7.7 | 2.7 |
| 2.5 | 8.69 | 10.3 | 3.6 | 0.77 | 9.0 | **3.1** |
| 2.9 | | | | 0.65 | 7.6 | 2.6 |
| 3.2 | 9.37 | 11.1 | **3.9** | 0.60 | 7.0 | 2.4 |
| 3.8 | 8.66 | 10.2 | 3.6 | 0.55 | 6.4 | 2.2 |
| 4.5 | 7.58 | 9.0 | 3.0 | | | |
| 5.0 | 5.32 | 6.5 | 2.2 | 0.40 | 4.7 | 1.6 |

* 0.005 n. solution.

brought about by means of 0.1 n. acetic acid and a mixture of 0.1 n. acetic acid (1 part) and 0.1 n. sodium acetate solution (4 parts). Table III will demonstrate that the enzymes are totally destroyed at 70°C. β-Galactosidase was more stable against heat at pH 3.2, where its optimum pH lay,

## Table III.

### Effect of Heating on the Galactosidases.

When incubated for 30 minutes at 37°C, the original solution hydrolyzed α-phenolgalactoside at pH 3.8 and lactose at pH 3.2 with $k \times 10^3$ of 3.2 and 6.9 respectively. Incubation with the heated enzymes was exerted under similar conditions.

| Temp. of heating (°C) \ pH of Heating | Substr. | α-Phenolgalactoside | | | Lactose | | |
|---|---|---|---|---|---|---|---|
| | | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k \times 10^3$ |
| 50 | 4.5 | 5.47 | 6.4 | 2.2 | 0.55 | 6.4 | 2.2 |
| | 3.2 | 4.53 | 5.4 | 1.9 | 0.75 | 8.6 | 3.0 |
| 60 | 4.5 | 2.36 | 2.8 | 0.9 | 0.45 | 5.3 | 1.8 |
| | 3.2 | 2.33 | 2.8 | 0.9 | 0.71 | 8.3 | 2.9 |
| 70 | 4.5 | 0.0 | | 0.0 | 0.0 | | 0.0 |
| | 3.2 | 0.0 | | 0.0 | 0.0 | | 0.0 |

* 0.005 n. solution.

than at pH 4.5. α-Galactosidase showed no such difference of resistance, in accord with expectation from being equally remote of the pH's of heating from that of optimum activity. If heated at this pH, more of it may survive at 60°, but, even then, the inactivation will be complete at 70°C, when judged from the extensive destruction observed at 60°C.

*Exp.* 3. Attempts were made to obtain solutions containing the galactosidases separately and a successful means was found out as follows.

β-Galactosidase solution: Portions of an original solution (the optimum pH of α-galactosidase was 3.8 and that of β-galactosidase 3.2) were mixed with an equal volume of various buffer solutions, shaken for 30 minutes with Kahlbaum kaolin (0.1 gm. per cc.) and centrifuged. The supernatant liquids were subjected to similar treatment repeatedly and from the successive supernatant liquids a part was taken, divided into halves and the pH was adjusted to 3.8 and 3.2 to see the power of attacking the substrates. Table IV will show that, by treatment twice with kaolin at pH 4.0, α-galactosidase was adsorbed off entirely but about half of β-galactosidase remained in the solution.

α-Galactosidase solution. 4 cc. of the original solution, same as above, were mixed with an equal volume of the buffer solution of pH 4.0, agitated for 30 minutes with 0.8 gm. of the kaolin and centrifuged. The centrifugate (C. I) and supernatant liquid (L. I) were separated.

C. I. was washed with changes of the same buffer solution, until no coloured substances came out into a washing (the final washing involved none of galac-

## TABLE IV.

### Adsorption to Kaolin of Galactosidases at Various pH's.

To measure enzymic activity, incubation at 37°C and pH's 3.8 (for α-galactosidase) and 3.2 (for β-galactosidase) was continued for 30 minutes. In digestion by the original enzyme solution of α-phenolgalactoside and lactose $k$'s multiplied with $10^3$ were 3.4 and 6.1 respectively.

| pH of shaking with kaolin | No. of supern. liquids / Substr. | α–Phenolgalactoside | | | Lactose | | |
|---|---|---|---|---|---|---|---|
| | | Decrease of thiosulf.* use (cc.) | Cleavage % | $k$x10³ | Decrease of thiosulf.* use (cc.) | Cleavage % | $k$x10³ |
| 3.2 | 1st | 0.0 | | | 0.09 | 1.1 | 0.4 |
| | 2nd | 0.0 | | | 0.0 | | |
| 4.0 | 1st | 5.09 | 6.0 | 2.1 | 1.00 | 11.8 | 4.2 |
| | 2nd | 0.0 | | | 0.77 | 9.0 | 3.6 |
| | 3rd | 0.0 | | | 0.34 | 4.0 | 1.3 |
| 5.0 | 1st | 6.79 | 8.1 | 2.8 | 1.13 | 13.2 | 4.7 |
| | 2nd | 0.65 | 0.8 | 0.3 | 0.77 | 9.0 | 3.2 |
| | 3rd | 0.0 | | | 0.35 | 4.1 | 1.4 |
| 6.0 | 3rd | 0.0 | | | 0.41 | 4.8 | 1.6 |

* 0.005 n. solution.

tosidases) and then the buffer solution of pH 7.0 added and stirred up. The centrifuged supernatant fluid was acidulated and removed from the appearing precipitate. The brown clear fluid obtained cleft α-phenolgalactoside and lactose to similar extents.

L. I (7.5 cc.) was again shaken with a new 0.8 gm. portion of the kaolin, and after washing of the centrifugate (C II) with 8 cc. of the same buffer solution, elution of the enzymes was tried at first with the buffer solution of pH 5.0, using three 3.25 cc. portions, and then with that of pH 7.0. The eluent of pH 5.0 remained non-coloured, but that of pH 7.0 became brown. Parts of the eluates were acidified to pH's 3.8 and 3.2 with 1% acetic acid and after centrifugation, the enzymic activity of the supernatant fluids was estimated. As is illustrated in Table V, the first eluate of pH 5.0 contained about 1/5 the amount of α-galactosidase in the original solution but was not free from the β. In the second eluate of the same pH was present no trace of the latter enzyme. Of the former only about 1/20 came over, but this solution will serve for structural study of galactosides, if long-timed, for instance 5 hours, diges-

## TABLE V.

### Elution from Kaolin (C II) at pH's 5.0 and 7.0 of the Galactosidases.

The activity of α-galactosidase was measured at pH 3.8 and that of β-galactosidase at pH 3.2. Incubation temperature, 37°C. Hydrolysis of α-phenolgalactoside and lactose by the original solution proceeded with k's multiplied with $10^3$ of 3.4 and 6.1 respectively in 30 minutes' digestion.

| Eluate pH | Eluate No | Time of incub. min. | Substr. α-Phenolgalactoside: Decrease of thiosulf.* use cc. | α-Phenolgalactoside: Cleavage % | α-Phenolgalactoside: $k \times 10^3$ | Lactose: Decrease of thiosulf.* use cc. | Lactose: Cleavage % | Lactose: $k \times 10^3$ |
|---|---|---|---|---|---|---|---|---|
| 5.0 | 1st | 30 | 1.73 | 2.1 | 0.7 | 0.0 | | |
| | | 120 | 6.96 | 8.2 | 0.7 | | | |
| | | 300 | 11.05 | 13.2 | 0.5 | 0.54 | 6.3 | 0.2 |
| | 2nd | 30 | 0.41 | 0.5 | 0.2 | 0.0 | | |
| | | 120 | 2.67 | 3.2 | 0.3 | 0.0 | | |
| | | 300 | 4.87 | 5.8 | 0.2 | 0.0 | | |
| | 3rd | 30 | 0.0 | | | 0.0 | | |
| 7.0 | 1st | 30 | 0.0 | | | 0.0 | | |

* 0.005 n. solution.

tion is applied (see the table). The eluate of pH 7.0 looked to have none of those enzymes.

The optimum pH's of the enzymes are probably shifted by treatment with kaolin so that the enzymic activity found at the original optimum pH's were not the maximum, but this possible inaccuracy is not estimated such as disproves the conclusion that the enzymes are obtained separately by adsorption to kaolin and elution therefrom at different pH's.

The experiments with α- and β-methylgalactoside revealed that these galactosides are respectively hydrolyzed much less readily than those α- and β-galactoside referred to, whereas the optimum pH's for the scission were not divergent.

Our thanks are due to the Grant Committee for Scientific Researches of the Education Department for a grant. H. Masamune.

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# Visual Function and the Electrical Excitability of the Retina.

By

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In his previous communication[1] the author reported that a subnormal electric stimulus leaves an excitatory state which subsides in a very peculiar manner, that is, in the form of a damped oscillation. Then arose the question, whether or not the same kind of excitatory state would be caused by optic stimulation.

It is a well known fact that optic stimulation gives rise to an electric change in the retina which is followed by action currents of the optic nerve. According to Adrian and Matthews,[2] Hartline and Graham[3] and others, the impulses of the optic nerve persist for a short time after the cessation of the stimulus. Therefore, we could expect that some change in the electrical excitability of the retina would be found not only during stimulation, but also after its termination. After-effects of visual stimulation have hitherto been investigated as after-images or as changes in the adaptation state, but never as changes of the electrical excitability. In the present investigation this new aspect of visual after-effects will be studied in detail.

## Method.

Electrodes, silver plates of $2 \times 2$ cm$^2$ in size were placed at the forehead and occiput and fixed by a bandage. The electrical contact was secured by means of a paste containing NaCl in high concentration. The stimulating current was a single rectangular pulse of 57 msec. duration. The circuit used in this experiment is illustrated in Fig. 1. $K_1$ and $K_2$ are contacts of Hashida's rheotome, and $R$ is a variable resistance of 1–10,000 ohms. A constant resistance $W$ in the circuit containing the head of the subject serves to hold the resistance of this circuit fairly constant.

The duration of the current has no effect at all on results, unless it is sorter than 50 msec. Currents of too short duration yielded qualitatively different results, but no mention is made of them in this report, for much space is required to explain the mechanism underlying this effect of duration.

The indicator of excitation used for the determination of thresholds was a light-sensation evoked by electrical stimulation. The sensation felt on such a weak stimulus as near the threshold was, however, so vague that its quality as light could not be discerned any more. In our experiment only the threshold for disappearance was measured, for the threshold for appearance was difficult to determine with the same accuracy as for the former. To reach the threshold, usually 10–20 trials had to be made, beginning with a current strong enough to elicit a distinct light-sensation.

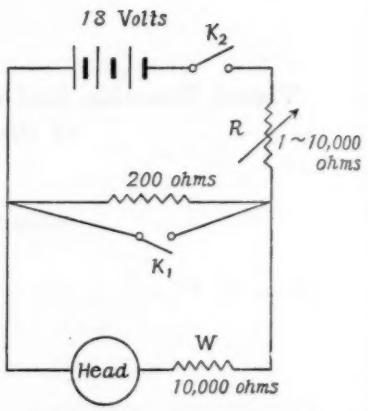


Fig. 1. Circuit for production of stimulating currents.

Sometimes the sound from the rheotome alone evoked a vague sensation difficult to distinguish from the sensation due to electrical stimulation, but it would disappear on repeated administration of the acoustic stimulus. I suppose it is a conditioned reflex. The conditioning, however, was no obstacle to our experiment, because our subjects soon learned to distinguish the sensation due to electrical stimulation from the conditioned one by the procedure for differentiation, that is, alternative administrations of the acoustic stimulus and that associated with the electrical stimulation.

Experiments were carried out in the dark after the subject had stayed in the dark for twenty minutes or so. The eyes were fixed by means of a fixation-point (a faint red light) so as to prevent eye-movements during stimulation. A disc of opaque glass was illuminated by an incandescent light from various distances, and viewed from a distance of 30 cm on the opposite side. The shutter was operated by the subject. The time of exposure was of little concern for the present investigation, but a too short exposure necessitated a considerably strong stimulus to have a distinct effect, so that the time of exposure of 1/5–2 sec. seemed to be suitable for ordinary arrangements of illumination.

The interval between illumination and electric stimulation was controlled manually under the aid of a metronome. Experiments were repeated once a minute for strong illumination, but for weaker light-stimuli

an interval of 30 sec. was sufficient to avoid any accumulation of successive effects.

At well exercised subjects, the threshold could be determined accurately to 2 per cent, and the reproducibility was so satisfactory that data obtained on different days showed good agreement with one another.

## Results.

### 1. The Time Course of the Change in Electrical Excitability after Illumination.

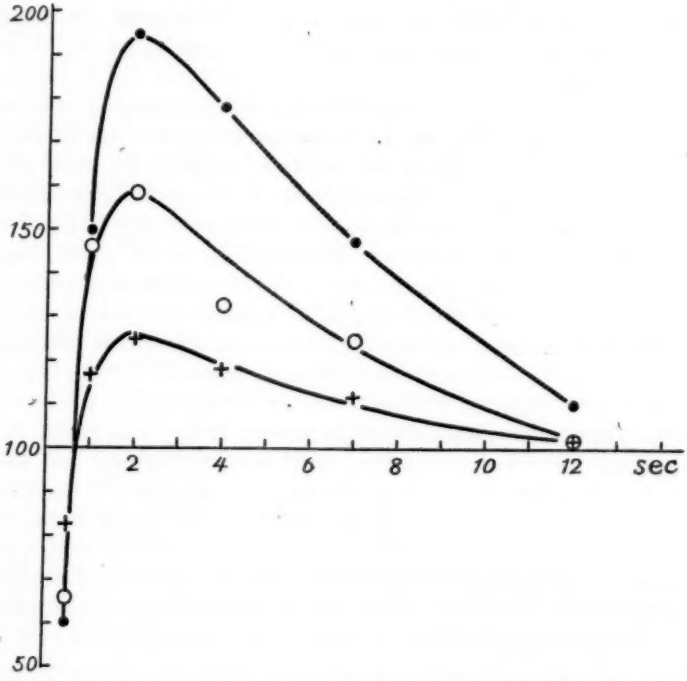


Fig. 2. Time course of electrical excitability of the retina after illumination. Duration of illumination was 1/5 sec. Intensities of illumination were 70, 1.1 and 0.06 lux for upper, middle and lower curves respectively. Ordinates: Reciprocal of electrical threshold in percentage of that before illumination. Abscissæ: Time after the end of illumination.

It was found that the electrical excitability of the retina considerably increases a few seconds after an illumination of the retina. The excitability curves for three different intensities, namely 70, 1.1 and 0.06 lux are illustrated in Fig. 2. In this figure the ordinate gives the reciprocal of the

threshold in percentage of that before illumination, and the abscissa the time after the end of the illumination. As seen from this figure, the excitability is subnormal immediately after the illumiation, but rises rapidly to become supernormal, reaches a maximum in about 2 seconds and decreases gradually. It is to be noted that the time to the maximum or " crest time " is almost independent of intensities of illumination. So long as white light was used the crest time was always about 2 seconds at the six normal subjects examined in our laboratory, and remained constant irrespective of the parts illuminated of the retina.

In spite of this constancy of the crest time, the height of the maximum was different from individual to individual, and depended greatly upon intensity and duration of light, area and region illuminated of the retina and other unknown factors.

The electrical excitability passes through a supernormal phase in this manner after an illumination, but such supernormality is never found when the excitability is tested with a second optic stimulus delivered after a first; the sensitivity to light is found always to be lowered after an illumination. Hecht[4][5] attributed this decrease in light-sensitivity to the decomposition of the photosensitive substances contained in the retina, and the restoration of light-sensitivity in the dark to the resynthesis of the substances from their decomposition products. It is possible that the supernormal excitability as determined electrically bears some relation to the decomposition products, for the properties of the excitability curve seem to support this view to some extent. For instance, the long duration of the supernormality suggests that some chemical process must be involved in this phenomenon. Electrical stimulation itself presumably does not involve cones and rods which are said to contain the photosensitive materials, because the after-effect or excitatory state left by an electric stimulus decays far more rapidly than the after-effect of an optic stimulus, as shown in my previous work. The difference in the time course of both sorts of excitability curves suggests the difference in the site of stimulation. Then, where is the seat of electrical stimulation, if it is not the photorecipient cells of the retina ?

The most probable seat is the ganglion cells of the retina for the following reasons: The fact that the nearer the electrode to the eye, the lower the threshold, indicates that either the retina or the optic nerve is electrically stimulated. If the optic nerve were the seat of electrical excitation, there should be the seat of interferance between the after-effect of an adequate stimulus and the effect of electric stimulation in cortical or subcortical visual centers. But the supernormality after illumination is of so long duration that it can hardly be understood as a process occurring in the higher centers, because according to Sherrington the excitatory state in

nervous centers as revealed by reflex enhancement is of much shorter duration. As mentioned above, there is a conspicuous difference between the after-effect of an adequate stimulus and that of an electric stimulus, and this fact cannot be explained from this stand-point, because it does not seem likely that nervous impulses set up by an adequate stimulus and those evoked by an electrical one would initiate qualitatively different excitatory states in the higher centers. Therefore, the seat of interferance must be the ganglion cells including their synapses in the retina. It is a very probable idea that photolytic products or their derivatives remain for a long time after illumination, and modify the electrical excitability of the ganglion cells in such a manner that they inhibit the electrical excitation in too high concentrations, but facilitate it in moderate concentrations. The subnormal phase found immediately after illumination may be due to an excess of the decomposition products.

In this connection it is necessary to refer to the result of an experiment, in which after an illumination of one eye the excitability of the opposite eye was measured. The one electrode was placed at the outer corner of the latter eye and the other above the brow on the same side. The result is shown in Table I. It will be seen from this table that at the unilluminated

TABLE I.

Effect of illumination of one eye upon the electrical excitability of the opposite eye.

| Time after illumination | 0.5″ | 1″ | 2″ | 4″ |
|---|---|---|---|---|
| Elect. excitab. of the opposite eye | 50 | 94 | 97 | 101 |
| Elect. excitab. of the illuminated eye | 43 | 120 | 200 | 145 |

eye only the subnormal phase, but no supernormal phase is found, while the same eye, when illuminated, shows the normal course of excitability. Since the representations of the fovea lie in both hemispheres, the illumination of the opposite eye would have brought about the same sort of excitability change, if the cortical center had been the seat of interferance. Therefore, this experiment, too, is inconsistent with the assumption of cortical summation. On the contrary, the fact that the excitability of the unilluminated eye was reduced by illumination of the opposite eye, suggests that the subnormal phase is partly due to the interferance occurring at the cortical level.

## 2. The Distribution of Electrical Excitability over the Retina.

The electrical excitability was measured two seconds after the illumination of various parts of the retina. The area illuminated was of 2° in diameter, and the intensity of illumination 287 lux. The result is shown in Fig. 3 (solid circles). As seen from this figure, the excitability of the retina is maximum at the fovea, and decreases gradually towards the periphery. The supernormal excitability vanishes, of course, at the blind spot. To be able to attribute this distribution of excitability to the local differences of the retina, we must be sure that it is independent of the arrangement of electrodes, for it is conceivable that at the arrangement of electrodes in this experiment the current would flow in the direction of the axis of the ganglion cells at the fovea, but obliquely to it at the periphery, so that the current would be most effective at the fovea only on account of its favorable direction of flow. To rule out this possibility, the electrodes were placed at temples so that the current flowed from right to left, namely perpendicularly to the current in the above experiment. The result is plotted in the same figure (empty circles in Fig. 3). As seen from this figure, the

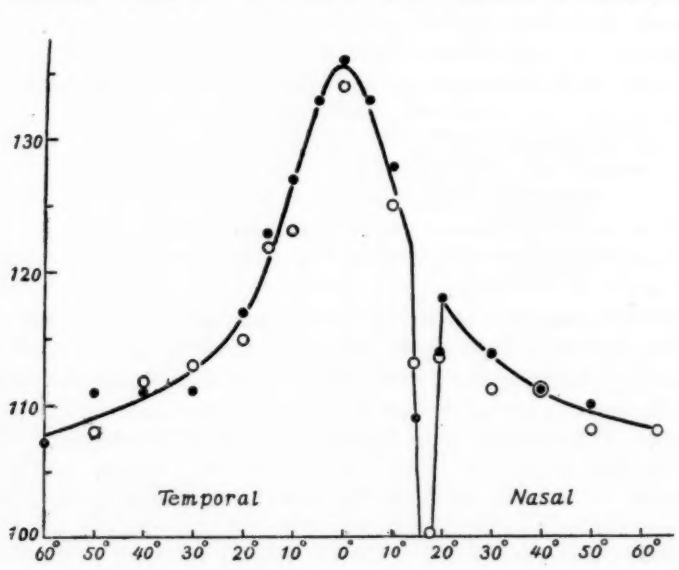


Fig. 3. Electrical excitability 2 sec. after illumination of various parts of the retina (left eye). Intensity of illumination was 287 lux. Data obtained with electrodes at forehead and occiput are represented by solid circles, and those obtained with bitemporal arrangement of electrodes by empty circles.

alteration of the arrangement of electrodes has brought about no alteration in the distribution of excitability at all. From this experiment we can safely conclude that our distribution curve represents the local differences in excitability of the retina.

However, it is still difficult to understand, why the result is independent of the arrangement of electrodes; it seems to be even against the laws of electrophysiology. So we must consider some possible reasons for this phenomenon in the following: The first possible explanation would be that the excitability of the ganglion cells of the retina would be independent of the direction of the current, but this is scarcely plausible unless more evidence is available, because it is known that the direction of current flow has a considerable effect upon the efficiency of excitation, so long as muscle and nerve are concerned. More plausible is the second explanation which is based upon the anatomical fact that the vitreus body of the eye is comparatively homogeneous and contains no cellular element. We know that a high resistance of tissues is chiefly due to the polarization at cell-membranes. As there is no cell-membrane at all in the vitreus body, the conductivity within the eye-ball must be higher than in its surroundings. It is supposed that under such circumstances the current traverses the retina almost perpendicularly to its surface, irrespective of the arrangement of electrodes in such a manner as illustrated in Fig. 4. The great size of electrodes used in our experiments must be one of the favorable conditions.

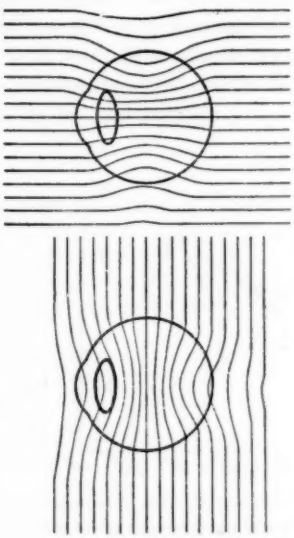

Fig. 4. Hypothetical stream lines at the eye-ball for both sorts of arrangement of electrodes.

It must be emphasized that this investigation has made it possible to explore electrically the properties of any desired part of the retina. Many attempts have hitherto been made to stimulate electrically any part of the retina by suitable adjustment of electrodes, but none of them has been successful, because it is impossible to place the electrode directly on the retina, and consequently to confine the current to any desired region. It is also difficult to determine subjectively the part of the retina stimulated electrically. In the dark, the subject feels a light-sensation in the periphery nearest to the cathode, but with such a weak stimulus as near the threshold the orientation of the light-sensation is so vague that nobody can give

any reliable information about the site of excitation. Now we can electrically stimulate any part of the retina we desire, if we previously sensitize that part with light.

The distribution curve in Fig. 3 shows a maximum at the fovea. This fact suggests that our measure must be related to the cone mechanism. There are few physiological measures which represent the excitation in the cones. Visual acuity is certainly one of such measures, but it is not clear how it is connected with the physiological process of the cones. The supernormal excitability after illumination is a measure surely connected with the cone excitation, so there is a prospect of utilizing this measure for researches on the cone mechanism.

### 3. The Electrical Excitability of the Retina and Twilight Vision.

Our measure concerns not only the cone mechanism, but also the rod mechanism. This is clearly shown in twilight vision, in which only rods are thought to be sensitized with light and consequently to become supernormal. In the following experiment various parts of the retina were sensitized with such a weak light as was scarcely visible by central fixation, and the electrical excitability was measured as usual. For comparison with the latter, also the sensitivity to light of these parts was measured by means of Nagel's adaptometer. The result is shown in Fig. 5. It will be seen from this figure that both curves agree very closely with each other. If the sensitivity to light depends on the amount of the photosensitive sub-

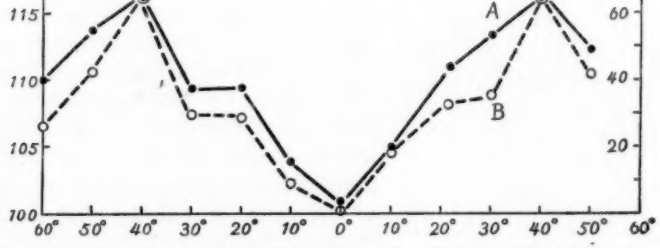


Fig. 5. Electrical excitability of the retina two seconds after the illumination with a white light of a definite low intensity corresponding to the threshold at the fovea (full line A) and the light sensitivity of the retina or reciprocal of visual threshold (broken line B). Abscissa: distance from the center of the retina in visual angle. Right: nasal side, left: temporal side.

stance, visual purple in this case, as Hecht pointed out, that must be the case also with the electrical supernormal excitability after illumination. If it is assumed that the electrical excitability of the ganglion cells in the retina is raised in proportion to the amount of the decomposition product of the visual purple the agreement between both sorts of curves will be at once realized, because the amount of the visual purple decomposed by a definite small quantity of light must be proportional to the amount present at that moment.

Summary.

The electrical excitability of the retina was measured in the dark with a light-sensation as an indicator.

1. It was found that any part of the retina, when sensitized with light previously, becomes more sensitive to electrical stimulation. The electrical excitability reaches a maximum about 2 seconds after illumination irrespective of its intensity.

2. For high intensities of sensitizing light the supernormal excitability is maximum at the fovea, and decreases towards the periphery.

3. For sufficiently reduced intensities of light the supernormality is minimum at the fovea, and maximum at 40° from the fovea. This distribution of the electrical excitability agrees with the distribution of the light-sensitivity over the retina.

These experiments suggest that the supernormal electrical excitability is an important measure applicable to researches on the mechanisms of daylight- and twilight vision.

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# The Relation between the Intensity of Light and the Electrical Excitability of the Human Retina.

By

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The electrical excitability of the human retina becomes supernormal after an illumination of the eye, as reported by Motokawa.[1] The supernormality can be determined with considerable accuracy under controlled conditions. This phenomenon depends upon properties of electrical stimulation, qualities and intensities of light, physiological states of the eye and other unknown factors. The most effective of these factors is certainly the intensity of illumination. The present investigation was undertaken in view of the fact that quantitative studies on the dependence of various visual phenomena on intensities of illumination have thrown considerable light on the mechanism of vision.

## Method.

As the details of the method used in this investigation have been described in Motokawa's paper cited above, only main points will be mentioned here. The experiment was commenced after the electrical threshold in the dark had become constant. Such a state of dark adaptation was reached usually in 20 minutes or so. After the determination of the electrical threshold in the dark, the eye was exposed to light of definite intensity for two seconds, and the electrical test-stimulus, a constant current pulse 100 msec. in duration was delivered two seconds after the end of the illumination. If the subject felt a light-sensation on electrical stimulation the voltage of the stimulating current was reduced step by step until the current no more elicited any sensation.

The trial was made every minute when the light stimulus was strong, but every 30 seconds with weaker illumination. The intensity of illumination was altered by varying the distance between the light source and a disc

of ground glass which was viewed by the subject on opening a shutter. Also a set of neutral tint filters served to change the intensity over a wide range.

## Results.

Before entering into the description of results we must refer to the meaning of the measure $\zeta$ which we use to express our results. If $E_0$ and $E$ represent the excitability or the reciprocal of threshold in the dark and that 2 seconds after the end of illumination respectively, $\zeta$ is defined as follows:

$$\zeta=\frac{E-E_0}{E_0}\times 100. \qquad (1)$$

Therefore, $\zeta$ is the excitability increase in percentage of the excitability in the dark. This measure depends little on physiologically insignificant conditions such as the unit of the electrical stimulus, the resistance of the circuit including the resistance of the head, etc., and expresses only the effect of illumination, a matter of the greatest interest in our investigation. On account of this merit we prefer $\zeta$ to other measures such as threshold voltage and excitability which are usually made use of, but not adequate to our purpose, because their values cannot be zero even when no light stimulus is given. The value of $\zeta$ increases beginning with zero as the intensity of illumination is raised over the threshold. In this respect our measure is comparable to the action current of the retina. We are measuring the magnitude of a certain photoreceptor process as a difference in electrical excitabilities.

### 1. $\zeta$-Log Intensity Relation at the Fovea.

The stimulating electrodes, silver plates of $2\times 2$ cm$^2$. in size were placed at the outer corner of the left eye and above the left brow, to confine the electrical stimulation to the left eye which was illuminated.

The data from two different subjects K. M. and K. I. are shown in Fig. 1, in which the excitability increase $\zeta$ is plotted against the logarithm of intensity. For convenience of illustration, the measurements for K. M. are displaced to the right 2 log units. The size of the test patch was 2° in diameter for K. I. and 8° for K. M., and in every case the patch was centrally fixated. It will be seen from this figure that the data fall on a smooth S-shaped curve, which has great similarity to the relation between the intensity of light and fusion frequencies of flicker or visual acuity in man and insects as studied by Hecht,[2] Hecht and Verrijp,[3] Wolf,[4] Hecht and Wald[5] and others. Hecht[6] showed that the data of flicker and other visual phenomena could quantitatively be described by the following equation:

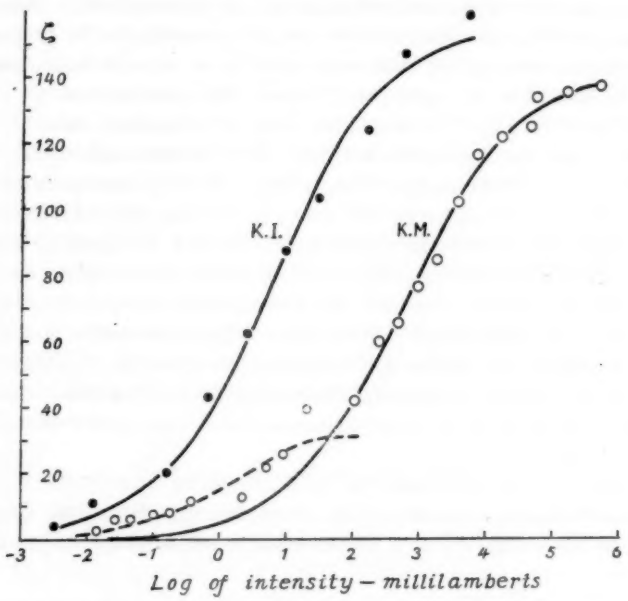


Fig. 1. Relation between ζ and log intensity of illumination at the fovea. Size of visual field was 2° for K. I. and 8° for K. M. For convenience in showing the result, the data for K. M. are displaced 2 log units to the right. Full curves are drawn with computed values after Hecht's equation. The part represented by a broken line belongs to the rod curve, which showed itself because the illumination was not confined to the fovea in this case.

$$KI=x^n/(a-x)^m. \qquad (2)$$

In this expression $I$ means the intensity of light, $x$ the concentration of the photolytic product in the stationary state, $a$ the initial concentration of the photosensitive substance, $K$ a constant, i.e. the ratio of the velocity constants of the light and dark reaction in the primary part of the photochemical system, and $m$ and $n$ are the numbers characterizing the order of the light and dark reaction respectively. It is of course necessary to make some plausible assumption to be able to relate experimental measures such as fusion frequencies of flicker and visual acuity to the concentration of the photoproduct $x$, and this was done by Hecht. Some of his assumptions were plausible, but others not so convincing.

As suggested in Motokawa's paper,[1] our phenomenon must be a secondary effect of illumination which is mediated by some chemical substance. The seat of the action of the chemical mediator is supposed to be synaptic layers of the retina. If this idea is correct it is no less adequate to

relate our measure to the concentration of the photo-product than fusion frequencies of flicker or visual acuity. So we assumed $\zeta$ to be proportional to the concentration $x$, and examined whether or not our data could adequately be described by equation (2) under this assumption.

The curve in Fig. 1 were drawn with so computed values of $\zeta$. It will be seen that the agreement between observed and calculated values is satisfactory over a wide range of intensities. The systematic deviation at low intensities for the curve K. M. is due to rod function which came into play, because in this case the illumination was not confined to the fovea. In this computation the constants of the formula were chosen as follows: $K=1.28$, $a=143$ for the data K. M., $K=1.28$, $a=155$ for the data K. I. and $m=n=2$ in both cases. With other values of $m$ and $n$ no such satisfactory agreement was attained. This situation is worth noticing, for also Hecht[6] could reproduce his data concerning flicker, intensity discrimination and other phenomena involving cone vision most accurately with the set of $m=n=2$.

In Hecht's work intensities of light are given in photons. This sort of unit, however, was not used in our work, because Stiles and Crawford[7] pointed out that visual effect of illumination is not proportional to the area

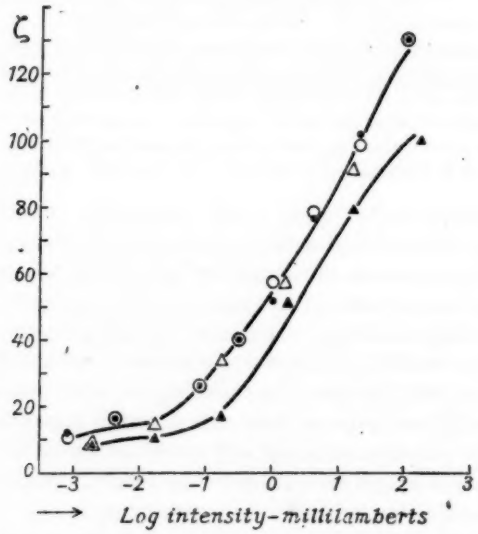


Fig. 2. Effects of pupilar size on $\zeta$—log intensity curves. Two series of measurements with natural pupil are expressed by empty circles and empty triangles respectively. Measurements with artificial pupils 3 mm. and 2 mm. in diameter are represented by solid circles and solid triangles respectively.

of the pupil. We have corroborated the findings of these authors with our own method. It is shown in Fig. 2 how much the value of $\zeta$ is reduced by using an artificial pupil of varying size. In this figure two series of data obtained with natural pupil on different days are expressed by empty circles and empty triangles respectively, to show the consistency of measurements. The data obtained with artificial pulils of 3 mm. and 2 mm. diameters are expressed by solid circles and solid triangles respectively. It will be seen that the pupil of 3 mm. diameter has no effect at all on $\zeta$, whereas the pupil of 2 mm. diameter has a somewhat remarkable effect.

As our subject stayed always in the dark except the time of illumination, his natural pupil must have been decidedly greater than 3 mm.—probably 7–8 mm.—, nevertheless the data with and without pupil control have completely coincided with each other. This fact indicates that the rays entering the pupil at peripheral parts is little effective compared with the paraxial rays.

## 2. $\zeta$—Log Intensity Curve at the Periphery.

Using the same sized field 2° in diameter we measured the relation between $\zeta$ and the intensity of illumination at 0°, 5°, 20° and 50° from the fovea. The result is shown in Fig. 3. While the data are represented by a single curve at the fovea, they fall on two clearly different curves. At low

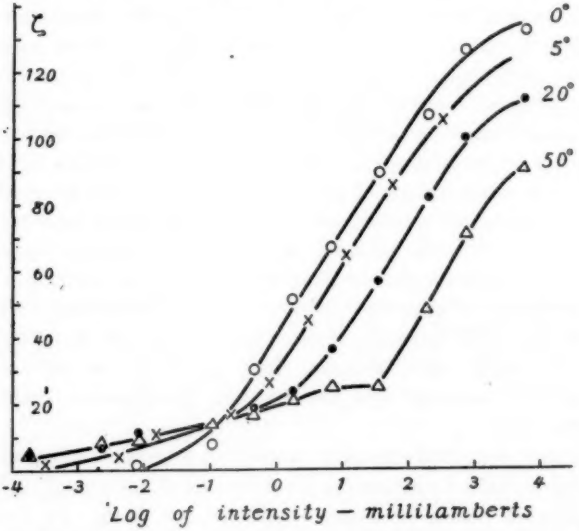


Fig. 3. $\zeta$—log intensity curves at 0°, 5°, 20° and 50° from the fovea. Visual field 2° in diameter.

intensities $\zeta$ rises with a gentle slope as log $I$ increases, and at a certain intensity the slope of rise abruptly becomes steeper. This critical intensity is higher as the measuring area moves from the center farther into the periphery. At extreme peripheries such as 40° and 50° $\zeta$ reaches a maximum which is maintained till an abrupt rise begins.

In the middle range of intensities the data lie with reasonable precision on a straight line, and the slope of the line is almost the same at every region of the retina. The dual nature of the peripheral measurements very likely represents rod function for the low intensity section and cone function for the high intensity section. As stated above, the two sections are separated more clearly as measurements are made farther and farther from the fovea.

The dependence of our data on light intensities greatly resembles the relation between critical fusion frequency of flicker and intensities of illumination, as studied by Hecht and Verrijp.[3] However, there is some difference between them; for instance, the high intensity section at 20° from the center reached a maximum at a much lower intensity in the experiment of these authors than in our own, and the value of the maximum was decidedly smaller than that of the curve obtained at the fovea. Though the intensity was not raised high enough to reach a maximum in our experiment, it is almost certain that the maximum is not so small as in the experiment of American authors.

In this regard some mention has to be made about the threshold determination in the periphery. In our experiment, there was, especially for inexperienced subjects, some difficulty in determining cone threshold at extreme peripheries, becuase the light-sensation due to cone excitation so feeble compared with the sensation due to rod excitation that the greatest care had to be taken so as not to be misled by predominant rod excitation. Otherwise, too small values of cone excitability would be obtained, and consequently the curve would bend at lower intensities to form a pseudo-maximum. Well trained subjects, however, was able to distinguish the sensation due to cone excitation from that due to rod excitation with considerable accuracy, so that two distinct values of threshold were obtained for one and the same intensity of illumination. From such subjects at all regions of the retina were obtained cone curves of the same slope which would completely coincide with one another, if adequately displaced along the intensity axis.

Although it is agreed that cone data from a number of experiments are adequately described by equation (2) when $m=2$, and $n=2$, it is not conclusively decided which equation applies to rod data. In Hecht's computation, the combination $m=2$, $n=2$ or $m=2$, $n=1$ showed a good fitness for visual acuity data, while the combination $m=2$, $n=1$ or $m=1$, $n=1$ were

TABLE I.

Validity of Hecht's formula $KI=\zeta^n/(a-\zeta)^m$ for rod-excitation.

| Intensity in millilamberts (blue light) | ζ obs. | ζ cal. | | Difference | | Square of difference | |
|---|---|---|---|---|---|---|---|
| | | m=2 n=2 | m=1 n=2 | m=2 n=2 | m=1 n=2 | m=2 n=2 | m=1 n=2 |
| 1.15 | 23 | 22.3 | 22.3 | —0.7 | —0.7 | 0.49 | 0.49 |
| 0.575 | 22 | 21.9 | 22.1 | —0.1 | 0.1 | 0.01 | 0.01 |
| 0.2875 | 22 | 21.2 | 22 | —0.8 | 0 | 0.64 | 0 |
| 0.0719 | 19.5 | 19.5 | 21.4 | 0 | 1.9 | 0 | 3.61 |
| 0.0359 | 16.5 | 18.2 | 20.6 | 1.7 | 4.1 | 2.89 | 16.7 |
| 0.01795 | 15 | 16.6 | 19.4 | 1.6 | 4.4 | 2.56 | 19.3 |
| 0.00288 | 13 | 11.5 | 13.1 | —1.5 | 0.1 | 2.25 | 0.01 |
| 0.00144 | 9 | 9.6 | 10.4 | 0.6 | 1.4 | 0.36 | 1.96 |
| 0.00072 | 9 | 7.7 | 8.5 | —1.3 | —0.5 | 1.69 | 0.25 |
| 0.00036 | 6 | 6 | 6.4 | 0 | 0.4 | 0 | 0.16 |
| 0.0000576 | 3 | 3.1 | 2.9 | 0.1 | —0.1 | 0.01 | 0.01 |
| 0.0000288 | 2.5 | 2.1 | 1.56 | —0.4 | —0.94 | 0.16 | 0.88 |
| | | | | | | Average | Average |
| | | | | | | **0.923** | **3.61** |

TABLE II.

| Intensity in millilamberts (white light) | ζ obs. | ζ cal. | | Difference | | Square of difference | |
|---|---|---|---|---|---|---|---|
| | | m=2 n=2 | m=1 n=2 | m=2 n=2 | m=1 n=2 | m=2 n=2 | m=1 n=2 |
| 1.185 | 23 | 23.4 | 23 | 0.4 | 0 | 0.16 | 0 |
| 1.296 | 21 | 22.9 | 22 | 1.9 | 1 | 3.61 | 1 |
| 0.0237 | 21 | 20.4 | 20.65 | —1.6 | —0.35 | 0.36 | 0.123 |
| 0.00529 | 16.5 | 17.6 | 18.2 | 1.1 | 1.7 | 1.21 | 2.89 |
| 0.000474 | 12 | 10.5 | 9.27 | —1.5 | 2.73 | 2.25 | 7.41 |
| 0.000118 | 5 | 6.7 | 5.32 | 1.7 | 0.32 | 2.89 | 0.102 |
| 0.00000948 | 5 | 2.4 | 1.61 | —2.6 | —3.39 | 6.76 | 10.41 |
| | | | | | | Average | Average |
| | | | | | | **2.46** | **3.13** |

TABLE III.

| Intensity in millilamberts (white light) | ζ obs. | ζ cal. | | Difference | | Square of difference | |
|---|---|---|---|---|---|---|---|
| | | m=2 n=2 | m=1 n=2 | m=2 n=2 | m=1 n=2 | m=2 n=2 | m=1 n=2 |
| 0.296 | 18 | 18.9 | 18.5 | 0.9 | 0.5 | 0.81 | 0.25 |
| 0.0237 | 18 | 16.4 | 17.5 | —1.6 | —0.5 | 2.56 | 0.25 |
| 0.00529 | 13 | 13.7 | 15.2 | 0.7 | 2.2 | 0.49 | 4.84 |
| 0.000474 | 8 | 7.6 | 7.25 | —0.4 | —0.75 | 0.16 | 0.563 |
| 0.000118 | 5 | 4.7 | 4.4 | —0.3 | —0.6 | 0.09 | 0.36 |
| 0.00000948 | 2.5 | 1.6 | 1.4 | —0.9 | —1.1 | 0.81 | 1.21 |
| | | | | | | Average | Average |
| | | | | | | **0.82** | **1.25** |

adequate for flicker data. In this manner the data were not sufficiently critical to allow a conclusive choice. We have made measurements at 40° from the fovea in three cases in order to determine the form of equation for rods. The results are shown in Tables I, II and III. It will be seen from these tables that the combination $m=2$ and $n=2$ is more adequate for our data than the combination $m=1$ and $n=2$, because the mean of squares of differences between calculated and observed values is in every case much smaller for the former combination than for the latter. The calculated values for other combinations of $m$ and $n$ showed so great deviations from the observed values that they were discarded from the tables. The constants chosen in the above computation were as follows: For the data in Table I, $K=323$, $a=23.5$, $m=2$, $n=2$ or $K=7300$, $a=22.3$, $m=1$, $n=2$, for the data in Table II, $K=1250$, $a=24.1$, $m=2$, $n=2$ or $K=14200$, $a=22.2$, $m=1$, $n=2$ and for the data in Table III, $K=766$, $a=20.2$, $m=2$, $n=2$ or $K=11600$, $a=18.6$, $m=1$, $n=2$.

If we choose the equation of the same form $KI=x^2/(a-x)^2$ for both cones and rods, the constant $K$ is considerably greater for rods than for cones, and the reverse is the case with the constant $a$.

## Discussion.

The duplicity theory of vision is so well-established that there seems to be no need to support this theory with further evidence. So the fact that our findings fall into the pattern of the duplicity theory may deserve no special attention. However it is important in having proved that our new method is an excellent method for studies of visual mechanisms. Since no visual sensation due to an adequate stimulus is used as criterion for measurements in our investigation, our method enables to study the mechanism of vision from an entirely different angle. In general, visual sensation involves not only the receptor process in the sensory elements, but also the nerve impulses and other changes in the central nervous system. In the interpretation of experimental results always some obscurity remains on account of the central process involved.

We have provided a number of evidence that the site of electrical excitationis is the ganglion cell layer of the retina, and that the modification of the electrical excitability must be mediated by some chemical substance. This view is supported above all by the fact that the supernormality due to illumination lasts for a considerably long time, for instance over 30 minutes after a long exposure to bright sun light. It is well known that the transmission of nervous impulses is mediated by some chemical substance such as acetylcholine or adrenaline. The excitatory state at synapses is considered to bear close relation to these substances. In our experiment, we are dealing with the excitatory state at the synapses of ganglion cells of

the retina, and the chemical mediater in this case must be some product of chain reactions initiated by the action of light. If the latter is produced in proportion to the concentration of the photo-product in the initial photochemical reaction, it is quite natural that Hecht's equation applies with great precision to our data.

For cone vision the order of reaction is bimolecular for both the light- and dark reactions, and eqation $KI=\zeta^2/(a-\zeta)^2$ is valid for all the cone data available and in our experiment also for rod data. As clearly seen from the form of this equation, the constant $K$ is independent of the unit of $a$ and $\zeta$, and the values of $K$ for cones and rods can be compared with each other if the light intensity is expressed in the same unit. In the subject K.M. the value of $K$ for rods was 1250, while that for cones was only 1.28, the former being 980 times as great as the latter. In other subjects the ratio was similarly high. $K$ is the ratio $k_1 : k_2$, where $k_1$ and $k_2$ mean the velocity constants for the photolysis and resynthesis in the dark respectively. Since $k_2$ may be regarded as inversely proportional to the time taken for complete dark adaptation, $k_2$ for cones must be several times as great as that for rods, because it takes 5–10 minutes for the dark adaptation of cones, but about 40 minutes for that of rods. Taking this ratio of $k_2$ into account, we must conclude that the velocity constant of the photolytic reaction in the rods would be several hundred times as great as that in the cones.

As shown above, the value of $a$ is much smaller for rods than for cones, for instance $a$ was 24.1 for rods, and 143 for cones in the same subject. From this fact it does not always follow that the initial concentration of the rod-substance is lower than that of the cone-substance, because the constant $a$ does not represent the concentration of the photosensitive substance itself. We have assumed that $a$ and $\zeta$ are proportional to the initial concentration of the photosensitive substance and to that of the photolytic product respectively, but made no mention at all about the proportionality factor. It is very probable that the latter varies with the kind of substance. The proportionality factor defines the efficiency of substances to elicit electrical disturbances. It suggests a higher efficiency of the cone-substance that the values of $a$ and $\zeta$ are much greater for cones than for rods in spite of the fact that the cone-substance has not yet been proved at least chemically.

## Summary.

The electrical excitability of the human retina becomes supernormal after an illumination of the eye. The maximum increase $\zeta$ in electrical excitability expressed in percentage of the excitability in the dark varies with intensities of illumination. The relation between $\zeta$ and log intensity is represented by a single sigmoid curve at the fovea, but consists of two

different sections outside the fovea.

The double nature of the peripheral measurements very likely represents rod function for the low intensity section and cone function for the high intensity section. The data for both cones and rods are adequately described by Hecht's equation $KI=\zeta^2/(a-\zeta)^2$.

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# Electrophysiological Studies of Color Vision.

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In spite of the vast literature on colors and color perception, the physiological mechanism of color vision has little been elucidated. We have so little knowledge of physiological processes directly concerned with color vision that we are hardly able to criticize many theories concerning this problem from the physiological standpoint.

Such phenomena as action currents of the retina, flicker sensation on intermittent optic stimulation were at first held promising in this respect and extensively studied, but no definite relation between hue and these phenomena has yet been established. So long as the brightness or luminosity of color is concerned, these phenomena are surely concerned with color, but have nothing to do with the hue.

In the preceding papers of Motokawa[1] and of Motokawa and Iwama,[2] it was shown that the electrical excitability of the human retina becomes supernormal after an illumination, and that the increase in excitability bears a quantitative relation to the intensity of illumination. When white light is used the maximum of supernormality is reached in about two seconds after the end of the illumination. In a preliminary experiment I have found that the time course of supernormal excitability varies as the color of light is changed. If this sort of variation is actually due to the difference of wave-lengths, we shall have a physiological phenomenon directly concerned with color vision, for we are sure that the increase in electrical excitability due to illumination is really a physiological event of the retina.

It is the object of the present investigation to decide the question conclusively whether or not our phenomenon is truely a physiological event directly concerned with the hue of color.

## Method.

After a stay of 20 minutes or so in the dark the electrical threshold of

the eye was determined with a constant current pulse 100 msec. in duration, the indicator being the faintest light-sensation evoked by the current. The stimulating electrodes were placed at the outer corner of the left eye and above the left brow. In one series of experiment the left eye was exposed to light for 2 seconds, but in another for 1/5 seconds, because in the former case a longer exposure was necessary on account of low intensities of monochromatic lights.

The excitability was measured at various intervals such as 0.5, 1, 2, 3,..... seconds after the end of illumination, these time intervals being controlled manually under the aid of a metronome. The visual field was 2° in diameter and centrally fixated by means of a fixation point (a faint red light).

The ranges of wave-lengths of colored lights yielded by the filters used in this experiment were 620–740 m$\mu$ for red, 490–550 m$\mu$ for green and 410–490 m$\mu$ for blue. In another series of experiment, monochromatic lights from a dispersion spectrum were used, the wave-lengths of which were calibrated by means of the line spectra yielded by sodium- and cadmium lamps.

The excitability increase due to illumination was expressed in percentage of the excitability in the dark before illumination and denoted by $\zeta$. For further details of the method I must refer the reader to the papers cited above.

## Results.

### 1. The Effect of Colors on the Time Course of the Excitability Curve of the Retina after Illumination.

Some examples of the data obtained with filters are shown in Fig. 1, in which the upper, middle and lower groups of curves were obtained from different individuals. As seen from this figure, the time course is most rapid for red, intermediate for green and slowest for blue i every case. It is interesting that this order is the same as the relative positions of the respective colors in the spectrum. The time course is characterized by the time to the maximum or crest time which is about one second for red, two seconds for green and about three seconds for blue. Since the crest time for white light is about 2 seconds, there seems to be no distinction between green and white lights in this respect. However, if the time course is examined in detail some differences between them will become apparent; for instance, the rise of curve is much steeper for green than for white.

It is worthy of note that the time course shows little individual differences, while the height of curves is different from individual to individual with one and the same intensity of light. I have made similar measure-

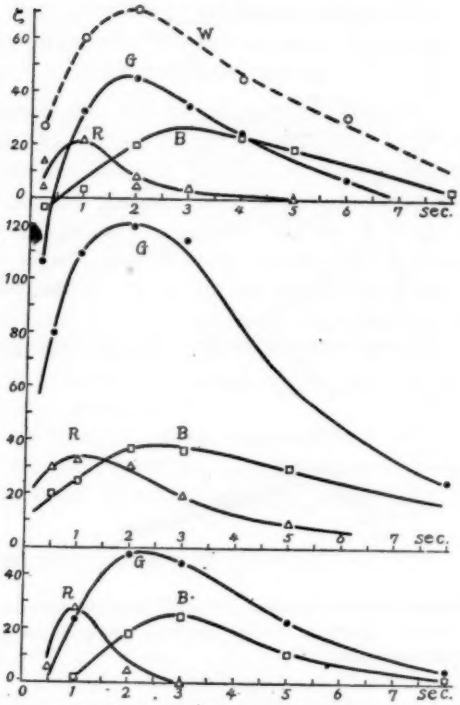


Fig. 1. Time course of supernormal electrical excitability of the retina after illumination of the eye by various colored lights. Exposure period 1/5 sec. Ordinate: Excitability increase in percentage of the excitability in the dark. Abscissa: Interval between the end of illumination and the electrical stimulation. W: white, R: red, G: green, B: blue.

ments in over ten different individuals and found that the time course was always the same for the same colored light with a few exceptional cases in which color vision was not normal. Researches on the deviation from normal in these cases are now in progress in our laboratory, so that the result will be reported in the near future.

The question now arises whether or not curves of the same type will be obtained at peripheral regions of the retina. Since these regions are known to be partially or completely color-blind, this question is of the greatest importance for deciding whether or not our phenomenon is really concerned with color vision. In Fig. 2 the result obtained at 40° from the center is illustrated. The data for the fovea of the same subject is shown also in this figure for the sake of comparison. At the periphery, the time

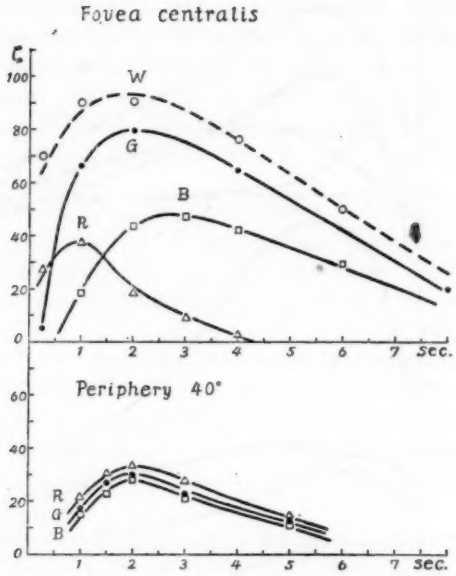


Fig. 2. Measurements at the fovea and at 40° from the center of the retina at one and the same subject. Curves at the periphery coincide with one another within the limits of experimental error.

course of curves for the three kinds of light shows no difference at all, what makes a striking contrast to the result for the fovea.

It must, however, be mentioned that at 40° from the center, curves can be different slightly from one color to another when the intensities are sufficiently high. This fact suggests that the degree of color-blindness of the periphery depends on intensities. The results at peripheral regions nearer to the fovea are similar neither to that at 40° from the center nor to that at the fovea, and too complicated to describe here briefly so that their communication must be postponed till next paper.

It is worth while noticing that the time course of the curves obtained at the periphery greatly resembles that of the curve for weak white light at the fovea. The crest time is about 2 seconds in both cases, and the shape of the curve is drawn out. A curve of the same type is obtained in the parafoveal region with such a weak light of any color as elicits only light-sensation but no color sensation. From these facts we can conclude that this sort of curve concerns the function of rods.

## 2. Has the Intensity of Light any Effect on the Time Course of Supernormal Excitability after Illumination?

It is well known that the brightness and hue of color correspond to the physical quantities, intensity and wave-length of light respectively. As stated above, other physiological phenomena such as the fusion frequency of flicker, the magnitude of the b-wave of the action current of the retina, etc. are related to the brightness of color, but not to the hue, because these measures can be controlled merely by varying the intensity.

In our phenomenon, the height of the maximum is probably some index of the brightness, for it varies as the logarithm of intensity over a wide range. Now it is of great concern whether the time course of supernormal excitability is solely determined by the wave-length of light, but not dependent on the intensity.

To answer this question, some measurements were made with the filters used in above experiments by altering intensities over a sufficiently wide range. The result obtained with the green light is illustrated in

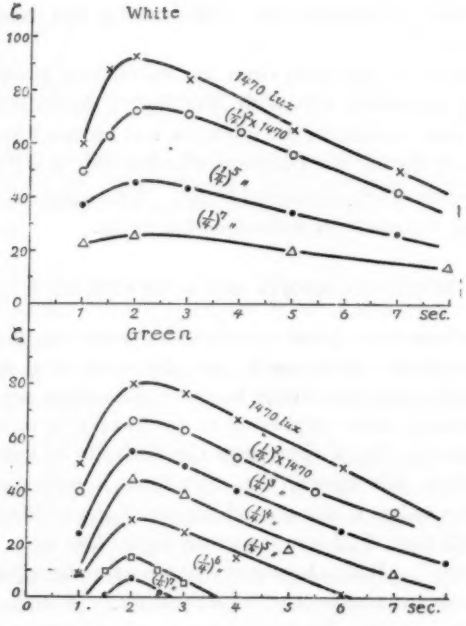


Fig. 3. Effects of light intensities on the supernormal electrical excitability of the retina after illumination by white and green lights.

Fig. 3. This shows that the position of the maximum undergoes no change at all by alteration of intensities. The curves for varying intensities run parallel with one another, so that at the lowest intensity which corresponds to the threshold of light sensibility, only the part near the maximum appears above the time axis. The relation, however, is somewhat different with white light; the curve becomes flatter with decreasing intensities, the crest time, however, remaining unaltered.

The flatness of the curve for white light is probably due to the circumstances that it contains not only green light but also red and blue lights which contribute to raise the initial and end parts of the curve. The crest time for the white light is determined chiefly by the green light contained in it. This is the reason why the crest time for white light coincides with that of green light. The fact that the height of the curve for green light is always greater than those of the curves for other colors, shows that the effect of green light is the determining factor for the crest time for white light.

The above mentioned flattning of the curve for white light with decreasing intensities may rest on the circumstances that the effect of green light decreases more steeply than do those of red and blue lights as the intensity is lowered. Experimental evidence for this relation will be reported elsewhere.

The constancy of the crest time was excellently proved also with red light, but found less satisfactory with blue light; the crest time in the latter case became a little shorter as the intensity was reduced near the threshold. It is important to decide the question whether this is a peculiarity to blue light or due to some other accidental cause. This question will be answered in the following experiments with spectral lights.

### 3. Measurements with Spectral Lights.

Above findings have been corroborated with much greater precision in these experiments. The results are shown in Fig. 4. The intensity at which the light was just visible by central fixation was taken as unity, and other intensities were expressed in terms of this unit. The number beside each curve in Fig. 4 represents the intensity of light.

As seen from this figure, the crest time is entirely independent of intensities. The value is 1.0 second for red light (650 m$\mu$), 1.25 sec. for yellow light (585 m$\mu$), 2.25 sec. for green light (530 m$\mu$), and 2.75 sec. for blue light (470 m$\mu$). It is to be noted that the crest time for monochromatic blue light is constant irrespective of intensities. It was elucidated in this experiment that when the illumination was not strictly confined to the rod-free part of the fovea, the excitation of rods could be predominant over

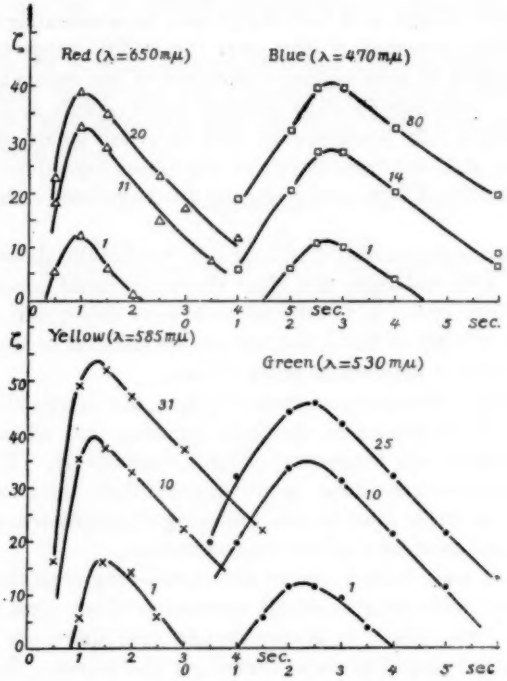


Fig. 4. Relation between the supernormal electrical excitability of the retina after illumination and the wave-length and intensity of spectral lights. The number beside each curve represents the intensity expressed in terms of threshold intensity at the fovea as unity.

that of cones at low intensities, so that the maximum could move towards that of the rod-curve. This happened more easily with lights of shorter wave-lengths, because they are more effective in exciting rods than lights of longer wave-lengths are. The above mentioned shift of the maximum for blue light is probably due to some fault concerning the fixation of the eye.

As seen from Fig. 4, curves for various intensities of one and the same colored light run almost parallel to one another. The intensity of light, therefore, serves only either to raise or to lower the curve as a whole, having no effect whatever on the time cours of the curve. This is a very obvious expression that the intensity- and time factors of our phenomenon are completely independent of each other. The process may accordingly be expressed as the algebraic sum of a function of time alone $F(t)$ and another function of intensity alone $\varphi(I)$. The form of $F(t)$ depends solely upon

the wave-length of light, and that of $\varphi(I)$ may approximately be represented by a logarithmic function at least over the middle range of intensities.

The function of time concerns the rate of the recovery process after illumination. The process must proceed more rapidly as the wave-length of light is longer. It is interesting that the time factor of light, i.e. the frequency controls the time factor of the physiological process involved, while the intensity of light concerns only the magnitude of the physiological process.

Now the question arises why blue light evokes the slower physiological process, and why red light can elicit the more rapid process, but cannot cause the slower one. It is difficult to answer this question with the experimental materials at hand, but not always difficult to suggest a possible explanation from a theoretical point of view.

According to the quantum theory of light, the energy of light is emitted or absorbed by multiples of the light quantum $h\nu$, where $h$ and $\nu$ are Planck's constant and frequency of light respectively. Therefore, lights of shorter wave-lengths have larger quanta than those of longer wave-lengths do. So there must be the following correspondence between light quanta and the crest time of our phhenomenon:

Short wave-length—large quantum—long crest time.

Long wave-length—small quantum—short crest time.

On the other hand, it is conceivable that there are some irritable systems with varying degrees of inertia in the retina. In a system with greater inertia, the process of excitation and recovery must proceed more slowly, what is characterized by a longer crest time in our experiment. Such a system will be activated only by lights of larger quanta, but never by those of smaller quanta, however strong their intensity may be, because the energy of activation depends solely upon the magnitude of quanta, but not on their number which is indicative of the intensity of light.

## Summary.

The electrical excitability of the human retina was measured by means of a constant current pulse 100 msec. in duration in the dark and after an illumination of definite duration and varying intensities. The increase in excitability due to illumination was expressed in percentage of the excitability in the dark before illumination, and plotted against the time interval between the end of illumination and the electrical stimulation.

1. The time course of so obtained curves varies with wave-lengths of light in such a manner that the crest time or time to the maximum increases as the wave-length decreases. The values of crest time for various colors are as follows: 1 sec. for red (650 m$\mu$), 1.25 sec. for yellow (585 m$\mu$), 2.25 sec. for green (530 m$\mu$) and 2.75 sec. for blue (470 m$\mu$).

2. The crest time is entirely independent of intensities of illumination.

3. Curves for varying intensities of one and the same colored light vary only in their height and run completely parallel to one another, indicating that the intensity- and time factors of this phenomenon are independent of each other.

## References.

(1) Motokawa, K., Tohoku J. Exp. Med., 1949, **51**, 145.
(2) Motokawa, K. and K. Iwama, Tohoku J. Exp. Med., 1945, **51**, 155.

The Tohoku Journal of Experimental Medicine, Vol. 51, Nos. 1 & 2, 1949.

# Biochemical Studies on Carbohydrates.

## CXIII. Hexosamine Compounds in Lung.

Second Report.

By

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(Received for publication, October 13, 1947)

The preparations in the first report[1] and those similarly obtained were subjected to quantitative analysis and optical measurement and their sugar components to identification by our usual method.[2] Fr. $A_1$ (Heparin) contained sulfuric acid and glucosamine in the molecular ratio of 2:1 as the heparin barium salt prepared by Masamune, Suzuki and Kondoh.[3] In the hemopoietic glycidamin (Fr. $A_2$) were found glucosamine, galactose and sulfuric acid in equimolecular proportions, and the group-specific glycidamin (Fr. $B_1$) possessed the same sugar components in the same proportion but was devoid of the sulfate rest.

## Experimental.

### Quantitative Analysis.

The quantitative figures are assembled in Tables I–III. The hemopoietic glycidamin is not perfectly soluble in plain water, but can be changed soluble if it is reprecipitated with alcohol after dissolution at pH 6.0 with the aid of alkali. The values in Table II are those of the preparations thus gained. Alkali used was $Na_2CO_3$.

### Identification of Amino Sugar and Hexose in the Both Glycidamins as Pentabenzoate and Methylphenylhydrazone Respectively.

1. The hemopoietic glycidamin. 1.5 gms. of this glycidamin were hydrolyzed and 84 mgms. of crude glucosamine pentabenzoate were obtained. By recrystallizations (5 times) from methanol till constant melting point (216°C.), the substance was reduced to 34 mgms. $[\alpha]_D^2$ in pyridine was $+44.84°\left(=\frac{0.31\times 3.0083}{0.0210\times 0.9901\times 1}\right)$. N(micro Kjeldahl), 2.04%. The

TABLE I.

Composition and Optical Rotatory Power of the Heparin Preparation (Fr. $A_{1a}$ in the First Report).

| Analysis | In per cent | In equivalents per equivalent weight |
|---|---|---|
| N* | 2.05 | 0.97 |
| Glucosamine | 27.67 | 1.00 |
| Glucuronic acid | | |
| Indole r. | 30.09 | |
| Orcinol r. | 30.45 | |
| Mean | 30.27 | 1.00 |
| Hydrolysable S | 9.23 | 1.86 |
| Ash | 24.13 | |
| $[\alpha]_D^{15}$ in water | $\frac{0.31\times 3.0250}{0.0258\times 1.005\times 1}=+36.37°$ | |

* Micro Kjeldahl.

TABLE II.

Composition and Optical Rotatory Power of the Hemopoietic Glycidamin.

| Analysis | In per cent | | | In equivalents per equivalent weight | |
|---|---|---|---|---|---|
| | Prep. I‡ | Prep. II | Average | Prep. I | Prep. II |
| N* | 13.53 | 13.77 | 13.65 | | |
| Glucosamine | 12.43 | 12.98 | 12.71 | 1.0 | 1.0 |
| Acetyl | 3.31 | | | 1.11 | |
| Galactose | | | | | |
| Indole r. | 12.74 | 13.49 | | | |
| Orcinol r. | 12.96 | 13.28 | | | |
| Diphenylamine r I., | 12.96 | 13.28 | | | |
| Mean | 12.89 | 13.35 | 13.12 | 1.02 | 1.02 |
| Hydrolysable S | 2.19 | | | 0.99 | |
| Ash† | 1.50 | | | | |
| $[\alpha]_D^4$ in water | $\frac{-0.59\times 3.0202}{0.0299\times 1.0036\times 1}=-53.39°$§ | | | | |

* Micro Kjeldahl. † Micro analysis. ‡ The substance in the first report. § Observed on Prep. I.

TABLE III.

Composition and Optical Rotatory Power of the Group-Specific Glycidamin.

| Analysis | In per cent | | | In equivalents per equivalent weight | |
|---|---|---|---|---|---|
| | Prep. I‡ | Prep. II | Average | Prep. I | Prep. II |
| N* | 12.41 | 13.02 | 12.72 | | |
| Glucosamine | 14.06 | 13.92 | 13.99 | 1.0 | 1.0 |
| Acetyl | 3.68 | 3.58 | 3.63 | 1.09 | 1.07 |
| Galactose | | | | | |
| Indole r. | 14.12 | 14.14 | | | |
| Orcinol r. | 14.35 | 14.25 | | | |
| Diphenylamine r I. | 14.00 | 14.00 | | | |
| Mean | 14.16 | 14.13 | 14.15 | 1.02 | 1.01 |
| Ash† | 3.35 | | | | |
| $[\alpha]_D^4$ in water | $\frac{-0.26\times0.9973}{0.0086\times1.0036\times1}=-30.04°$§ | | | | |

*Micro Kjeldahl. †Micro analysis. ‡The substance in the first report. §Observed on Prep. I.

methylphenylhydrazone separated at first was 62 mgms. After recrystallizing from 30% alcohol three times, this melted at 189–190°C. Further purification with pyridine did not raise the melting point, and when it was mixed with galactosemethylphenylhydrazone, no depression of melting point occurred. It did not rotate D-light in pyridine. N (micro Dumas) was found 9.95% (Calc. for $C_{13}H_{20}O_5N_2$, 9.85%).

II. The group-specific glycidamin. 1.0 gm. of this glycidamin was taken with the aim in view. Three times recrystallized, the benzoate showed constant melting point and weighed 18 mgms. F. p., 216°C. $[\alpha]_D$ in pyridine $=\frac{0.30\times1.0013}{0.0068\times0.9901\times1}=+44.62°$. N (micro Kjeldahl), 2.02%. Galactosemethylphenylhydrazone obtained (the crude, 42 mgms.) was similarly purified as above. F. p., 189–190°C. Mixed melting point test showed no depression. No optical rotation in pyridine. N (micro Dumas) found, 9.84%.

Thanks are due to the Grant Committee for Scientific Researches of the Education Department for a grant in aid to us. H. Masamune.

**Bibliography.**

1) Utusi, Tohoku J. Exp. Med., this volume, p. 101.
2) Cf. Satoh et al., ibid., 1943, **46**, 189, and Tiba, ibid., 1949, **50**, 281.
3) Masamune, Suzuki and Kondoh, J. Bioch., 1940, **31**, 343.

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# Spatial Summation of Optic Stimuli in the Human Retina as Revealed by Electrical Stimulation.

By

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(Received for publication, October 14, 1947)

There is much evidence which tends to show that spatial summation takes place in the human retina. For instance, there is an inverse relation between threshold intensity and size of area illuminated (Piper,[1] Reeves,[2] Piéron[3]). Beitel[4] presented two semi-circles separated by various distances and determined the intensity threshold as a function of the distance between the two stimuli. The result shows that when both semi-circles are presented together the threshold intensity increases as the stimuli are taken farther and farther apart up to the limit of separation of 2°30′, beyond which no interaction takes place any more.

As to the summation of over threshold stimuli, there are the experiments of Granit[5] and of Granit and Harper.[6] Granit determined flicker fusion frequencies for four circular test patches together and for each of the patches alone. With a higher intensity of illumination the fusion frequencies for all the patches were regularly 2.5 revolutions per sec. on an average higher than those of single patches at 10° from the center. With central fixation there was much less summation under otherwise similar conditions. Granit and Harper found a linear semi-logarithmic relation between flicker fusion frequencies and area illuminated, and the slope of the curve to be a little steeper in the periphery than in the center. From these results Granit concluded that summation takes place in the periphery to a greater extent than in the center.

Motokawa[7][8] has shown that the effect of light can be measured quantitatively by measuring increases in electrical excitability of the retina after illumination. Motokawa and Iwama[9] have demonstrated that the relation between the maximum increase of electrical excitability due to illumination and intensities of light is very similar to the relation between flicker fusion frequencies and intensities of illumination as studied by

Hecht and Verrijp.[10] The present research has been undertaken to investigate spatial summation in the retina with this new method introduced by the author.

## Method.

The details of the procedure involved are described in Motokawa's papers cited above so that they may be omitted in this report.

Large silver electrodes were placed at the outer corner of the left eye and over the left brow, the electrical contact between skin and electrodes being made with electrode paste of high NaCl concentration. A constant current pulse 100 msec. in duration was used as a test-stimulus, and the threshold was determined with light sensation due to electrical stimulation as the index. Measurements were performed in a darkened room after complete dark adaptation of the eye. The maximum of electrical excitability is usually reached in two seconds after the end of illumination when white light is used. So only the threshold at this moment was measured. arrangements for illumination will be described later.

Let the electrical excitability or the reciprocal of threshold in the dark be $E_0$, and the excitability measured two seconds after the end of illumination be E, then the excitability increase $\zeta$ is defined as follows:

$$\zeta = \frac{E - E_0}{E_0} \times 100.$$

All the following results will be expressed in terms of $\zeta$.

## Results.

### 1. Summation at Varying Distances between Stimuli.

Such an arrangement of test patches as indicated in Fig. 1 (A) was used in this series of experiment. Each patch had a diameter of 1° visual angle, and the distance $x$ between the central and peripheral circles was varied between 0° and 6°. The central patch was always centrally fixated. The intensity of illumination was 1000 lux and the period of exposure 0.13 seconds.

The value of $\zeta$ was determined for the central patch alone and for all five together. The result is reproduced in Table I. The value of $\zeta$ for all the patches together is decidedly greater than that for the central patch alone at the fovea and parafoveal regions. Summation is weaker as the distance $x$ increases, and no indication of interaction is seen at a separation of 6°. So the limit of separation can be estimated at about 5° for this intensity of illumination. In order to see how the limit of separation varies with intensities of illumination, similar measurements were made with intensities 5200, 280 and 180 lux at another subject, and the follow-

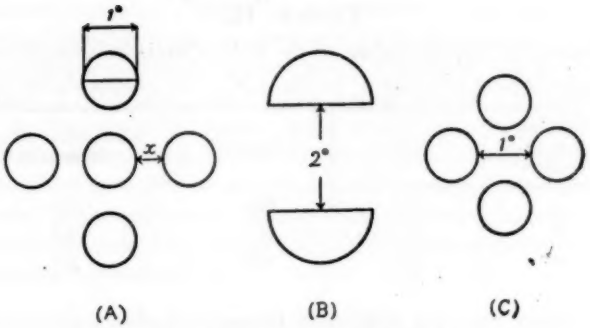


Fig. 1. Arrangements of test patches.

ing values were obtained: 13.6° for 5200 lux, 7° for 820 lux and 3.5° for 180 lux. Granit found fairly distinct summation in his "four-spot" flicker experiment with a separation of 2° and an intensity of 100 lux. It seems,

TABLE I.

Summation effects at varying degrees of separation between stimuli (five-spot experiment).

$x$ represents the nearest distance between the central and the other circles (arrangement of patches in Fig. 1 (A)). Intensity of illumination: 1000 lux. Exposure period: 0.13 seconds.

| $x$ in degrees | "Single" | "Five" | Summation effect (difference) |
|---|---|---|---|
| 0 | 54.5 | 77.0 | 22.5 |
| 1 | " | 75.5 | 21.0 |
| 2 | " | 68.0 | 13.5 |
| 3 | " | 64.0 | 9.5 |
| 4 | " | 58.5 | 4.0 |
| 6 | " | 54.5 | 0 |

therefore, that the order of magnitude for the limit of separation agrees in both cases, though the experimental methods are quite different from one another.

## 2. Summation at Various Parts of the Retina.

Summation at various regions of the retina was examined with the arrangement of test patches shown in Fig. 1 (B). The result is shown in Table II. At the center, the value of $\zeta$ is 63.5 for the single semi-circle and 69 for both semi-circles together, the difference being 5.5. The values of $\zeta$ for singles and doubles decrease as the location moves farther and farther

TABLE II.

Summation at various regions of the retina (semi-circle experiment).

Distance between semi-circles: 2° (Fig. 1 (B)). Illumination: 1000 lux.

| Location (degrees from the center) | "Single" | "Double" | Summation effect (difference) |
|---|---|---|---|
| 0 | 63.5 | 69.0 | 5.5 |
| 20 | 50.0 | 55.5 | 5.5 |
| 40 | 42.0 | 48.0 | 6.0 |

from the center, but the difference between double and single remains almost constant. If the difference is a faithful measure of summation the above result indicates that there is no local difference concerning summation. But the values of $\zeta$ decrease from the center towards the periphery so that the percentage increase of $\zeta$ by doubling the area must be greater for the periphery than for the center. The question as to which of these alternative interpretations is correct will be discussed in the following section.

This result has further been substantiated by another series of experiment which was performed with the arrangement of stimuli as illustrated in Fig. 1 (C). The result is reproduced in Table III. The values of $\zeta$ decrease as the measuring area moves from the center farther into the periphery, but the difference between the value for all the four patches together and that for the single varies only little like the result of above experiment.

TABLE III.

Summation at various regions of the retina (four-spot experiment).

Arrangement of patches in Fig. 1 (C). Illumination: 1000 lux.

| Location (degrees from the center) | "Single" | "Four" | Summation effect (difference) |
|---|---|---|---|
| 0 | 41.0 | 68.5 | 27.5 |
| 10 | 39.0 | 62.5 | 23.5 |
| 20 | 35.0 | 58.5 | 23.5 |
| 30 | 21.5 | 47.0 | 25.5 |
| 40 | 17.5 | 39.0 | 21.5 |
| 50 | 13.5 | 31.0 | 17.5 |
| Illumination: 8 lux. | | | |
| 0 | 15.5 | 37.0 | 21.5 |
| 10 | 14.0 | 23.5 | 9.5 |
| 20 | 15.5 | 15.5 | 0 |
| 30 | 8.0 | 6.0 | −2.0 |
| 40 | 6.0 | 6.0 | 0 |

In the experiment with a much lower intensity of illumination some indication of summation is seen at the fovea and at 10° from the center, but in the farther periphery there is no indication of summation at all. As shown in the report of Motokawa and Iwama, values of $\zeta$ for rods are generally much lower than those for cones; the former seldom exceed 30. Therefore, it is difficult to show distinctly the summation for rod excitation in the periphery. The fact that it has failed to prove any summation with the low intensity in the periphery, does not always mean that no summation takes place in the periphery, but only shows that the summation of peripheral cone excitation is weak or fails.

### 3. The Dependence of $\zeta$ on Area Illuminated.

The relation between $\zeta$ and area was quantitatively studied at the fovea and in the periphery. The size of patches was varied from 0.2° to 3° in diameter. The intensity of illumination was 1000 lux. The results are represented graphically in Fig. 2.

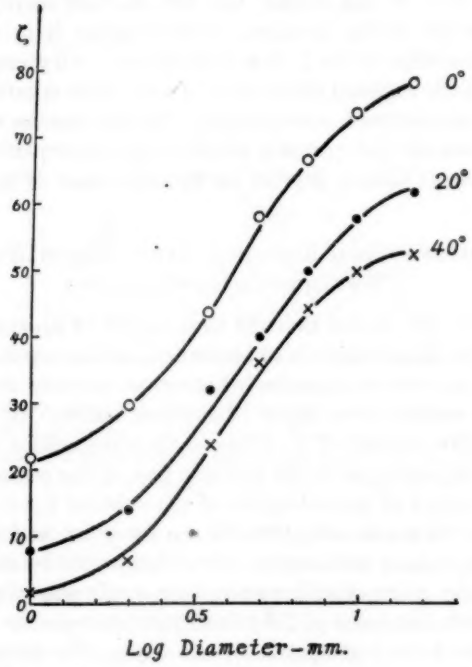


Fig. 2. Relation between the excitability increase $\zeta$ due to illumination and the logarithm of area illuminated. Intensity: 1000 lux. Period of exposure: 0.13 seconds.

As seen from this figure, the shape of curves is always sigmoid just like the relation between $\zeta$ and log intensity as studied by Motokawa and Iwama.[9] In the experiment of Granit and Harper, fusion frequencies of flicker were in a linear relation to the logarithm of area, and the slope of curves was steeper in the periphery than in the center. However, in my experiment the curves for the fovea and for the periphery (20° and 40° from the center) run almost parallel with one another, and in the middle range of area in which a linear relationship approximately holds, the slope of the curves is almost the same for the fovea and the periphery. Thus, four experimental results seem to be inconsistent with those of Granit and Harper.

In the middle range of areas a geometric increase in area corresponds to an arithmetic increase in $\zeta$. So a definite amount of increase in $\zeta$ will be obtained by enlarging the area illuminated in a definite proportion, no matter what the initial value of $\zeta$ may be. This relation gives some clue to the question whether the absolute or relative (percentage) increase of $\zeta$ should be taken as a measure of summation. As stated in the preceding section, the percentage increase in $\zeta$ as the result of summation is greater in the periphery than in the center, but the absolute increase remains unaltered irrespective of the location. This relation is in entire harmony with the contsant slope of the $\zeta$—log area curves. All these results indicate that there are little regional differences, if any, with regard to summation, so long as our phenomenon is concerned. Further studies will be necessary to be able to answer the question whether the discrepancies between my results and those of Granit depend on the difference of methods or some other causes.

## 4. Interaction between Areas Illuminated by Various Colored Lights.

Motokawa[8] has shown that the time course of supernormal electrical excitability after illumination is characteristic of the wave-length of light. It is of great interest to examine whether or not any interaction occurs between areas activated by lights of different colors. In this experiment two semi-circular patches of 2° diameter in juxtaposition were used so as to confine the illumination to the rod-free part of the retina (Fig. 3). The approximate ranges of wave-lengths of the colored lights from the filters used were 620–740 m$\mu$ for red, 490–550 m$\mu$ for green and 410–490 m$\mu$ for blue. So long as both semi-circles were illuminated by one and the same colored light the interaction between both areas was always marked for any sort of color, the value of $\zeta$ for both patches together being decidedly higher than that for any one patch viewed alone. For instance, $\zeta$ increased from 42 to 75 by doubling the area with red light, from 43 to 71 with green light and from 45 to 69 with blue light. On the contrary, there was no

indication of summation at all when one semi-circle was illuminated by the red light and the other by the blue light.

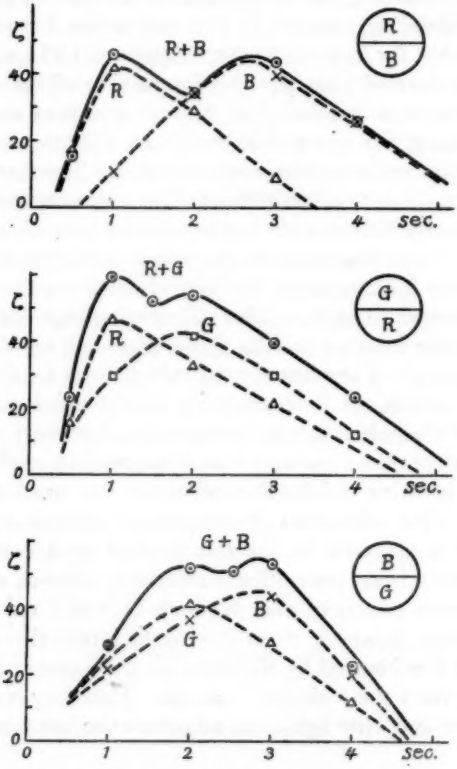


Fig. 3. Interaction between colored fields (semi-circles in juxtaposition).
Abscissa: Time interval between optic and electric stimulation.
Ordinate: Percentage increase in electrical excitability after illumination.
Full lines: Summation curves. Broken lines: Curves for single patches.
R, G and B mean red, green and blue lights respectively. The arrangement of the semi-circular patches is shown in the upper right corner of each figure.

The time course of the excitability curves for the "singles" and "doubles" is illustrated in Fig. 3 (the uppermost group of curves). As seen from this figure, the summation curve, R+B which was obtained by simultaneous presentation of red (R) and blue (B) semi-circles does not deviate greatly from the "single" curves, R and B.

The middle group of curves in the same figure shows the interaction between areas excited by green and red lights. In this case the sum-

mation curve, R+G is higher than any one of the " single " curves, indicating that there is some interaction between the red and the green semicircle. The increase in $\zeta$ due to summation is about 13 and much smaller than the corresponding amount in the summation between areas of one and the same color, for instance 33 for red light and 28 for green light.

Between green and blue areas an interaction of the same order as in the previous case was proved (the bottom group of curves in Fig. 3). Generally speaking, the more akin the colors of both areas to each other, the more striking the interaction between them. The fact that there takes place little interaction between red and blue areas suggests that both sorts of light must involve different kinds of mechanisms which act independently of each other. Some degree of interaction proved between red and green or green and blue indicates that the pair of lights must call into play some mechanism common to each. After all, our findings fall into the scheme of one of the color theories such as those advanced by Young and Helmholtz or by Hering. They assume that there must be three or four kinds of mechanisms which act independently of one another.

If Young-Helmholtz's theory is adopted to interpret our findings, the scant interaction between red and blue areas may be explained as follows: The red light involves chiefly R-component, but scarcely B-component, while the blue light stimulates B-component without involving R-component. As it is assumed in the theory that each component acts independently, there is no reason for interaction between the field in which only R-component is active, and the area in which only B-component is active. In reality, however, there is a slight interaction between red and blue area, what is evidenced by the fact that the summation curve, R+B is slightly higher than the " single " curves. This may rest on the circumstances that red and blue lights can stimulate the component G to a slight degree.

The interaction between red and green areas or green and blue areas may be ascribed to the properties of the green light to stimulate not only G-component but also R- and B-component to some extent.* In this way, we are led to the conception that summation or interaction occurs only among components or mechanisms of one and the same kind.

It is also worth while noticing that the theory of Young-Helmholtz applies to our findings which have been obtained without resort to any color sensation.

## Summary.

Spatial summation in the human retina was studied with increases of electrical excitability by illumination as the index.

1. The maximum increase in electrical excitability is reached in 2

seconds after an illumination. This amount was expressed in percentage of the electrical excitability in the dark and denoted by $\zeta$. The value of $\zeta$ after an exposure to five circular patches 1° in diameter was always higher than the value for a single patch of the same size. The closer the five patches to one another, the greater the value of $\zeta$.

2. Two semi-circles separated by an unstimulated region of 2° was used to study summation at various parts of the retina. The value of $\zeta$ was greater by a constant amount for both semi-circles together than for a single semi-circle irrespective of the location in the retina.

3. $\zeta$-log area curves were sigmoid in shape and their slope was the same in the fovea and in the periphery. These findings suggest that there are little regional differences with regard to summation.

4. Two semi-circular patches in juxtaposition were employed to investigate the interaction between colored lights. Summation was most striking between same colored areas, and fairly marked between red and green fields or green and blue areas, but there was no indication of summation at all between red and blue areas. The result was explained on the basis of Young-Helmholtz's theory of color vision.

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* To interpret this phenomenon, physiological induction should further be taken into account, as has been reported by Motokawa and Iwama in a preliminary report (this journal, 1949, **50**, 292).

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# Vacuum Desiccation of B. C. G. in Frozen State.

By

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Efforts to actively immunize against tuberculosis have been so numerous that it is quite impossible to do justice to the large literatures in a short summary. But we can distinguish them into three general methods which include the administration of killed organismus, living attenuated bacilli and living virulent bacteria in minute doses. The most extensive investigation for human beings had been made with attenuated living bacteria, especially with B.C.G. We have immunized more than 4,000,000 persons with the B.C.G. vaccine, which we have prepared in our institute. However, the B.C.G. vaccine, used for the prevention against tuberculosis at present, can not be preserved longer than one week without loss of its potency. Moreover it must always be kept in container of low temperature.

These facts are the greatest obstacles for the popularization of the inoculation of B.C.G. vaccine. We have tried to prepare the dried B.C.G. vaccine in vacuum from the frozen state to remove these faults that is to say to prepare a vaccine of equal effectiveness for a long time.

At first we attempted to make clear the conditions of preparing and preserving the dried B.C.G. vaccine.

### *Influence of Low Temperature upon B.C.G.*

If low temperature of freezing kills B.C.G. and weakens their activities as antigen, this procedure must be avoided. Galtier (1) maintains that tubercle bacilli do not die on account of cold. According to Calmette (2) tubercle bacilli neither die nor lose their vitality in low temperature (about −180°C) by liquid air or liquid nitrogen. In our experiment, B.C.G. at the end of the second week of the third generation on the Rockemann's culture medium, to which transplanted B.C.G. from glycerol bile potato

medium, were emulsified by means of cold agate mortar and diluted with physiological saline solution or desillated water to let one c.c. of the emulsion contained 5 mg. or 10 mg. B.C.G.

B.C.G. emulsion was frozen by employing the ammonia gas refrigerator, kept in −8°C for two hours, −10°C, −15°C, −20°C, or −30°C for half an hour respectively, melted again in room temperature, mixed by shaking and diluted with physiological saline solution in the arithmetic progression. B.C.G. of the diluted emulsion ($5\times10^{-2}$—$5\times10^{-8}$ mg) was transplanted on Oka & Katakura's culture medium (3). After one month of cultivation, there could be found no remarkable difference of colonies between the control emulsion and the frozen. (s. Table I)

TABLE I.

Influence of Low Temperature.

| No. | Medium | Temp. | Time | Dilution and Number of Colonies | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $5\times10^{-2}$ mg | $5\times10^{-3}$ mg | $5\times10^{-4}$ mg | $5\times10^{-5}$ mg | $5\times10^{-6}$ mg | $5\times10^{-7}$ mg | $5\times10^{-8}$ mg |
| I | Physiol. Saline Solution | Control | | ||| ||| ||| ||| 20 | 1 | 0 |
| | | −8°C | 2 hrs. | ||| ||| ||| ||| 7 | 1 | 0 |
| II | 〃 | Control | | ||| ||| ||| ||| 30 | 4 | 0 |
| | | −30°C | 30min. | ||| ||| ||| ||| 5 | 2 | 0 |

(|||) Beyond Count. (||) Over 50.

When B.C.G. emulsion was frozen in liquid nitrogen for five minutes, half an hour, two hours, four hours, eighteen or twenty-four hours respectively, the number of colonies decreased a little, but was not much different from the control emulsion. (s. Table II) As seen from the experiments de-

TABLE II.

Influence of Low Temperature (with Liquid Nitrogen).

| No. | Medium | Temp. | Time | Dilution rnd Number of Colonies | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $10^{-1}$mg | $10^{-2}$mg | $10^{-3}$mg | $10^{-4}$mg | $10^{-5}$mg | $10^{-6}$mg | $10^{-7}$mg |
| I | Distilled water | Control | | ||| ||| ||| ||| || 3 | 0 |
| | | −195°C | 5min. | ||| ||| ||| ||| 7 | 0 | 0 |
| II | 〃 | Control | | ||| ||| ||| ||| || 3 | 0 |
| | | −195°C | 30min. | ||| ||| ||| ||| 12 | 1 | 1 |
| III | 〃 | Control | | ||| ||| ||| ||| 12 | 1 | 0 |
| | | −195°C | 2 hr. | ||| ||| ||| ||| 5 | 0 | 0 |
| IV | 〃 | Control | | ||| ||| ||| ||| 12 | 1 | 0 |
| | | −195°C | 4 hr. | ||| ||| ||| ||| 4 | 0 | 0 |
| V | 〃 | Control | | ||| ||| ||| ||| || 24 | 0 |
| | | −195°C | 18 hr. | ||| ||| ||| ||| || 5 | 1 |
| VI | 〃 | Control | | ||| ||| ||| ||| || 24 | 0 |
| | | −195°C | 24 hr. | ||| ||| ||| ||| 35 | 5 | 0 |

scribed above, the decrease of colonies is not proportional to the degree of low temperature and the time of freezing.

*Transition of the Temperature of the Material to be Dried in the Ampoule during Vaccuum Desiccation in the Frozen State.*

The vacuum desiccating apparatus, which we have used, is a self-made desiccator of Flosdorf-Mudd's type (4), provided 30 attachments for ampoules. Hygroscopic reagent is dried $Cacl_2$. A rotary oil pump was used and its minimum pressure attains to $3 \times 10^{-4}$ mm. Hg. and its velocity of exhalation is 25 L. per minute. The temperature in ampoules during vacuum desiccation has close relation with its effectiveness. To study this relation we kept a little thermometer in an ampoule, which contained B.C.G. emulsion, during desiccation.

As medium of emulsion 2% pepton solution was used. One c.c. emulsion with 5 mg. B.C.G. was poured into an ampoule and was frozen in −20°C for half an hour. Ampoules were kept in an ice-box to keep the temperature below 10°C during desiccation. Before attachment of the ampoules to the apparatus, the pressure in it was 1/100 mm. Hg. The pressure was measured by Brunner's vacuum-gauge. Transitions of temperature and pressure in the ampoules are shown in a schedule as follows.

| Time | Temperature (C) | Pressure (mm.HG) | Notice |
|---|---|---|---|
| at Time of Attachment | −20° | 1/100—3 | |
| 5 min | − 1° | 2 | Bubbled a little from the frozen material. |
| 8 〃 | − 1° | 1 | Frost appeared on the wall of ampoule. |
| 12 〃 | − 1° | 1/10 | No more bubble. State of automatic freezing. |
| 15 〃 | − 1° | 1/20 | |
| 60 〃 | − 8° | 1/100 | |
| 2 hr. | − 8° | 1/100 | Volume of material decreased a little. |
| 3 〃 | − 8° | 1/100 | |
| 4 〃 | − 8° | 1/100 | One part of material like a spiderweb, another part like the inside of bread. |
| 5 〃 | − 7° | 1/100 | Frost on the wall changed partly to ice or dew. Desiccation seems to be almost completed. |
| 6½ 〃 | 0° | 1/100 | Desiccation finished. |
| 7 〃 | +10° | 1/100 | Dew on wall disappeared. |
| 8 〃 | +10° | 1/100 | Desiccation completed. |

When the room temperature is high or the degree of vacuum is lowered, automatic freezing can not be perfect. The material bubbles, becomes gelatinous, and not powdered by shaking after desiccation. Such "dried B.C.G." showed bad results of cultivation and preservation. Therefore, when temperature exceeds 15°C, I always employed the ice-box to cool the ampoules below 10°C.

*Preservation of " Dried B.C.G. Vaccine " in Room Temperature.*

The principal purpose in preparing " dried B.C.G. vaccine " by vacuum desiccation in the frozen state is to lengthen its effective period. In low temperature, either " dried B.C.G. vaccine " and " not-dried B.C.G vaccine " are effective for a fairly long time.

Hayashi[5] desiccated " B.C.G. vaccine " by vacuum desiccation in the frozen state, preserved it between 0°C and 5°C for five months. The number of the living bacteria was not decreased remarkably compared with those of fresh B.C.G. vaccine just after preparation. Shitama[6] observed that in the immunity test on the guinea pigs there was no difference between the " frozen dried B.C.G vaccine," kept in the ice-chamber for a full month, and the " non-dried B.C.G. vaccine." Cohn[7] could preserve tubercle bacilli living for three years after vacuum desiccation.

The same experiment was performed by us. At first I observed that $5 \times 10^{-5}$ mg. of not-dried B.C.G. vaccine develops 40 colonies after 42 days of preservation in an ice-chamber (0–5°C), when 5 mg. B.C.G. was contained in 1.0 c.c. emulsion. (s. Table III) Another part of the emulsion

TABLE III.

Experiment of Preservation of Undried B.C.G. in an Ice Chamber (0—5°C).

| Days of Preservation \ Dilution Grade | Medium : 2% Pepton | | | | | | |
|---|---|---|---|---|---|---|---|
| | $5\times10^{-2}$ mg | $5\times10^{-3}$ mg | $5\times10^{-4}$ mg | $5\times10^{-5}$ mg | $5\times10^{-6}$ mg | $5\times10^{-7}$ mg | $5\times10^{-8}$ mg |
| 1 | ||| ||| ||| ||| 19 | 0 | 0 |
| 28 | ||| ||| ||| 30 | 1 | 0 | 0 |
| 42 | ||| ||| ||| 40 | 0 | 0 | 0 |

was kept in room temperature (5–18°C). The surface of the emulsion was covered by liquid paraffin to avoid the contact of air, and preserved for 2 months, shaded from sunlight. After 29 days one colony was found from $10^{-5}$ mg. B.C.G. and after 2 months 2 colonies from $10^{-3}$ mg. B.C.G. (Table IV)

" Dried vaccine " which was prepared under the conditions above mentioned, was put into a dark box, and preserved in room temperature for six months in spring and summer. As seen from Table V, there could scarcely be found any difference of living bacteria of B.C.G. during three months but after six months the number of colonies is one tenth of the number just after the desiccation. Colonies decreased further to one tenth then B.C.G. were preserved for thirteen months. Instead of 2% pepton,

TABLE IV.

Experiment of Preservation of Undried B.C.G. in Room-temperature.

| Days of Preservation \ Dilution Grade | Medium : Physiological Saline Solution | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^{-1}$ mg | $10^{-2}$ mg | $10^{-3}$ mg | $10^{-4}$ mg | $10^{-5}$ mg | $10^{-6}$ mg | $10^{-7}$ mg |
| 1 | ||| | ||| | ||| | ||| | 5 | 2 |
| 29 | ||| | ||| | ||| | 5 | 1 | 0 | 0 |
| 60 | ||| | 5 | 2 | 0 | 0 | 0 | |

TABLE V.

Experiment of Preservation of Dried B.C.G. in Room-temperature.

| Month of Preservation \ Dilution Grade | $5\times10^{-2}$ mg | $5\times10^{-3}$ mg | $5\times10^{-4}$ mg | $5\times10^{-5}$ mg | $5\times10^{-6}$ mg | $5\times10^{-7}$ mg | $5\times10^{-8}$ mg |
|---|---|---|---|---|---|---|---|
| Just after Drying | ||| | ||| | ||| | ||| | 31 | 2 | 0 |
| 1 Month | ||| | ||| | ||| | ||| | 18 | 1 | 0 |
| 2 ,, | ||| | ||| | ||| | ||| | 15 | 0 | 0 |
| 3 ,, | ||| | ||| | ||| | ||| | 21 | 0 | 0 |
| Preservation in a Dark Box. Medium: 2% Pepton | | | | | | | |
| Just after Drying | ||| | ||| | ||| | ||| | 9 | 1 | 0 |
| 6 Months | ||| | ||| | ||| | 14 | 1 | 0 | 0 |
| 13 ,, | ||| | Saplophyten | 25 | 1 | 0 | 0 | 0 |
| Preservation in a Dark Box. Medium: Bouillon | | | | | | | |
| Just after Drying | ||| | ||| | ||| | ||| | 7 | 0 | 0 |
| 6 Months | ||| | ||| | ||| | 1 | 1 | 0 | 0 |

5% milk sugar, 5% cane sugar, 5% glucose, 1% dextrine, 5% serum and Rockemann's solution were also used to prepare dried vaccine. These dried vaccine were placed in room temperature for six months, including summer, and no bacteria was cultivated from them.

But as we see in Table V, two times diluted bouillon can be used as well as 2% pepton solution.

Considering the results above described, pepton was the most suitable

medium, which was used because of the following advantages: a) harmless to the life of bacteria, b) easily desiccated, c) not easily gelatinous even at the time, when freezing a little incomplete, and vacuum is not strict, d) easily dissolved again, and become fast homogeneous, and e) easily obtainable material.

*Influence of Temperature on the "Dried B.C.G. Vaccine."*

Corper & Cohn[8] observed that of 19 species of human and bovine tubercle bacilli, which were cultivated on glycerol broth, sealed in ampoules and preserved in 37°C for twelve years, twelve species were capable of growing.

"Ampoules oi dried B.C.G.", which were prepared under the same condition above mentioned, were put in the incubator of 37°C keeping their vacuum state and then B.C.G. were cultivated on Oka & Katakura's medium. As seen in Table VI, the number of colonies do not decrease in six weeks, but after ten weeks colonies lessened markedly. From the results above mentioned, "dried B.C.G.", which was desiccated by skilful technique, bears up against transportation or preservation in summer.

TABLE VI.

Experiment of Preservation of Dried B.C.G. in 37°C.

| Days of Preservation \ Dilution Grade | $5\times10^{-2}$ mg | $5\times10^{-3}$ mg | $5\times10^{-4}$ mg | $5\times10^{-5}$ mg | $5\times10^{-6}$ mg | $5\times10^{-7}$ mg | $5\times10^{-8}$ mg |
|---|---|---|---|---|---|---|---|
| 30°C 1 Day | ||| ||| ||| || 27 | 3 | 0 |
| ,, 6 Weeks | ||| ||| ||| || 13 | 1 | 1 |
| ,, 10 Weeks | ||| || || 5 | 0 | 0 | 0 |

*Influence of Direct Sunshine on "Dried B.C.G. Vaccine."*

"Dried B.C.G." were exposed to sunshine for definite time and then cultivated on the medium. Such experiment on B.C.G. can not be found in literature, but the researches about the influence of sunlight and ultraviolet on tubercle bacilli are numerous. Koch[9] found in 1890 that tubercle bacilli on the medium suffering from direct sunshine die within several minutes to several hours, according to the thickness of the bacilli membrane. Ogawa[10] exposed various kinds of tubercle bacilli emulsions in a quarz glass test tube to sunshine; by exposure to sunshine for fourteen minutes on a fine winter day and in summer for nine minutes, all bacilli died. Straus[11] and Treskenskaja[12] observed that tubercle bacilli were killed

by exposure to sunshine for several hours.

Two ampoules of "dried B.C.G.", both prepared under the same condition were provided. The one of them was exposed to direct sunshine and the other was envelopped with black paper and shaded from sunshine. The ampoules were made of ordinary glass. Living B.C.G. were examined by cultivation. Temperature in sunshine was from 14° to 18°C.

After exposure to the sunshine in 16°C for one or two hours, the number of colonies is not different from the control. In case of exposure for three or four hours, the number of colonies decreased a little, but not so much. After five hours, the number of colonies decreased a little. By exposure to sunshine for ten hours, the number of colonies decreased considerably.

TABLE VII.

Influence of Direct Sunshine.

| Hours of Sunshine \ Dilution Grade | $5\times10^{-2}$ mg | $5\times10^{-3}$ mg | $5\times10^{-4}$ mg | $5\times10^{-5}$ mg | $5\times10^{-6}$ mg | $5\times10^{-7}$ mg | $5\times10^{-8}$ mg |
|---|---|---|---|---|---|---|---|
| 1 (15°C) | ||| ||| ||| ||| 20 | 2 | 0 |
| 1 (Control) | ||| ||| ||| ||| 18 | 1 | 0 |
| 5 (14°C) | ||| ||| ||| ||| 4 | 0 | 0 |
| 5 (Control) | ||| ||| ||| ||| 20 | 2 | 0 |
| 10 (15°C) | ||| ||| ||| ||| 26 | 4 | 0 |
| 10 (Control) | ||| ||| ||| ||| ||| 18 | 4 |

Three ampoules of the same "dried B.C.G." were provided.

The first ampoule was in the vacuum state.

The second ampoul was cut at its neck and was open to the air.

The third ampoule was cut at its neck and was filled with one c.c. of physiological saline solution to make again an emulsion.

The three ampoules stood for three hours, exposed to the sunshine.

Temperature was 18°C. After exposure, B.C.G. were cultivated to examine the number of colonies.

The first and the second ampoules obtained almost similar number of colonies. But no colony was found in the case of the third ampoule. (s. Table VIII).

As above mentioned, "dried B.C.G. vaccine," we prepared, can bear up against the exposure of sunshine in a temperature below 18°C, and the exposure to the sunshine for a short time, which may take place by uncareful treatment, brings no influence upon B.C.G.

TABLE VIII.

Influence of Direct Sunshine.

| 3 Hours Sunshine \ Dilution Grade | $5\times10^{-2}$ mg | $5\times10^{-3}$ mg | $5\times10^{-4}$ mg | $5\times10^{-5}$ mg | $5\times10^{-6}$ mg | $5\times10^{-7}$ mg | $5\times10^{-8}$ mg |
|---|---|---|---|---|---|---|---|
| 1 in Vacuum | ||| | || | 20 | 1 | 0 | 0 | 0 |
| 2 Unsealed | ||| | || | 20 | 5 | 0 | 0 | 0 |
| 3 Diluted again | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

But B.C.G. in the state of emulsion die by direct exposure to the sunshine or three hours.

## CONCLUSION.

1) " B.C.G." at the end of the second week on Lockermann's medium was frozen in the low temperature of between $-8°$ and $-195°$C, but no remarkable influence was found. Freezing bears no influence upon " B. C.G." as the previous treatment of the vacuum desiccation in the frozen state.

2) If the temperature of air rises at the time of desiccation, the ampoule must be cooled below 10°C, in order to have a good result.

3) Pepton is the most suitable medium of " dried B.C.G."

4) " Dried B.C.G.", which employed pepton as the medium, can bear up against the exposure of sunshine for several hours.

But B.C.G. in the " emulsion " must be shaded from sunshine and be inoculated within a short time.

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# A Physiological Basis of Color Discrimination.

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All the observations on the sensibility of the eye to wave-length differences which give rise to differences in hue, have hitherto been made on the basis of color sensation; color sensation has been the only indicator with which all the problems of color vision have been investigated.

Color is a sensation felt on stimulation by light of some wave-length, but the latter is no immediate cause of color sensation, for its properties can be entirely different according to the conditions of the eye. There is undoubtedly a sequence of physiological processes between color sensation and light stimulation, but we are very little informed of these intermediate physiological processes, although they are variously pictured in the theories of color vision.

So long as color sensation is used as the only indicator for this sort of measurements it seems difficult to establish the physiological ground for color vision. The author has reported in his preceding papers[1][2] that the time course of the electrical excitability of the eye after illumination is characteristic of the wave-length of the light used, and that this phenomenon is very probably a physiological event occurring in the synaptic layer of the retina. If this phenomenon is made use of, it is possible to investigate the sensibility of the eye to wave-length differences without resort to any color sensation. In the present communication it has been attempted to elucidate the physiological mechanism of color discrimination by means of the electrical method introduced by the author.

## Method.

*Electrical measurements.* Electrodes consisting of silver plates $2 \times 2$ cm$^2$ in size were placed at the outer corner of the left eye and above the left brow, the electrical contact being made with electrode-paste rich in NaCl. The stimulating current was a constant current pulse 100 msec. in duration

which was yielded by the electrical circuit illustrated in Fig. 1 of the preceding paper.[2] The indicator for threshold determination was the slightest light-sensation due to electrical stimulation. It was of no great concern in this research what sort of color was felt on electrical stimulation.

The central part of the eye (2° in diameter) was exposed to a spectral light of certain wave-length for two seconds, and at various intervals after the end of illumination the electrical threshold was determined. To reach the threshold, it was in usual necessary to repeat the stimulation procedure —illumination followed by electrical stimulation—ten to twenty times, beginning with a voltage high enough to elicit a distinct light-sensation. The interval between each trial was 30 seconds. Measurements were always performed in a darkened room after complete dark adaptation. The difference between values of electrical excitabilities (reciprocals of threshold) with and without preceding illumination was expressed in percentage of the latter and denoted by $\zeta$.

*Determination of chromatic sensibility of the eye.* For this purpose, two general methods have been used. The first involves the comparison of two contiguous homogeneous fields by the method of least perceptible difference in wave-length or mean error, and the second consists in determining the width in wave-lengths of a portion of the spectrum which is shortened until it appears monochromatic to the subject. In the present investigation the second method was used.

The spectrum was projected on to a ground glass in front of the subject, and its wave-length was read from a micrometer scale projected as well. The scale was calibrated by means of the line spectra from a cadmium- and a sodium lamp. In order to avoid any stray light, the above mentioned ground glass which constituted a small window of a closed compartment, was viewed by the subject sitting in it. The investigator standing outside the compartment narrowed a slit in front of the ground glass step by step until the light appeared monochromatic to the subject and read the width of the slit in terms of wave-lengths. Measurements were made at intervals of 10 m$\mu$ in wave-lengths, proceeding in one set of tests from the red end of the spectrum to the violet end, and in another set in the reverse order.

Laurens and Hamilton[3] emphasize that one of the most evident factors influencing hue discrimination is that of the relative luminosity of the two wave-lengths. It was found in my experiment that the luminosity difference between the two parts of the patch was so marked especially in both end portions of the spectrum that it was difficult to decide whether the perceived difference was due to either difference in hue or that in luminosity. So it was attempted to minimize this sort of error by reducing the luminosity difference by means of an adequate gray wedge.

## Results.

### 1. Curves of Discrimination Sensibility for Hues of the Spectrum.

This sort of researches has been made by a number of investigators such as König and Dieterici[4], Uhthoff,[5] Brodhun,[6] Steindler,[7] Jones,[8] Edridge-Green,[9] Laurens and Hamilton[3] and others. Of these works Steindler's data are the first complete, and the data of Laurens and Hamilton are probably the best available at present. These measurements have shown that hue discrimination is at its best in the yellow and blue-green, and that in the orange and violet there are two secondary minima of threshold wave-length difference. Although the general form of the relationship between threshold difference $\Delta\lambda$ and wave-length $\lambda$ does not differ greatly among normal persons, the precise positions of the threshold minima vary surprisingly from person to person even under identical conditions. Therefore, it is necessary to make optical and electrical measurements on one and the same subject. Our data concerning hue discrimination may be not so accurate as those of Laurens and Hamilton, because the effects of luminosity are not completely ruled out, but they have the advantage that

TABLE I.

The minimum perceptible difference of wave-length $\Delta\lambda$, the crest time $\tau$ of electrical excitability curve and $\Delta\lambda/\Delta\tau$.

| $\lambda$ (mμ) | $\Delta\lambda$ (mμ) | $\frac{\Delta\lambda}{\Delta\tau}\times 10^{-2}$ (mμ. sec.$^{-1}$) | $\lambda$ (mμ) | $\tau$ (sec.) |
|---|---|---|---|---|
| 420 | — | 5.70 | 405 | 3.15 |
| 430 | 11.0 | 1.96 | 424 | 3.12 |
| 440 | 9.0 | 0.80 | 442 | 3.00 |
| 450 | 6.0 | 0.40 | 451 | 2.80 |
| 460 | 2.7 | 0.70 | 460 | 2.60 |
| 470 | 3.6 | 2.50 | 479 | 2.55 |
| 480 | 2.7 | 2.50 | 499 | 2.40 |
| 490 | 1.0 | 1.10 | 516 | 2.35 |
| 500 | 2.2 | 2.10 | 533 | 2.35 |
| 510 | 2.8 | 3.70 | 553 | 2.00 |
| 520 | 6.0 | 4.70 | 570 | 1.50 |
| 530 | 8.5 | 5.60 | 590 | 1.15 |
| 540 | 5.0 | 1.00 | 606 | 1.10 |
| 550 | 4.6 | 0.30 | 630 | 1.05 |
| 560 | 4.6 | 0.40 | 650 | 1.00 |
| 570 | 2.3 | 0.50 | 670 | 1.00 |
| 580 | 0.9 | 0.90 | | |
| 590 | 0.7 | 1.00 | | |
| 600 | 1.7 | 1.10 | | |
| 610 | 4.6 | 2.70 | | |
| 620 | 7.2 | 5.60 | | |
| 630 | 6.9 | 5.60 | | |
| 640 | 11.4 | 3.00 | | |
| 650 | 15.5 | 6.30 | | |

TABLE II.

The minimum perceptible difference of wave-length $\Delta\lambda$, the crest time $\tau$ of electrical excitability curve and $\Delta\lambda/\Delta\tau$.

| $\lambda$ (mμ) | $\Delta\lambda$ (mμ) | $\frac{\Delta\lambda}{\Delta\tau}\times10^{-2}$ (mμ. sec.$^{-1}$) | $\lambda$ (mμ) | $\tau$ (sec.) |
|---|---|---|---|---|
| 410 | — | 7.30 | 410 | 3.00 |
| 420 | — | — | 430 | 2.95 |
| 430 | 8.8 | 1.54 | 450 | 2.65 |
| 440 | 6.7 | 0.48 | 470 | 2.55 |
| 450 | 4.8 | 1.11 | 495 | 2.50 |
| 460 | 2.8 | 1.88 | 505 | 2.30 |
| 470 | 4.3 | 2.60 | 516 | 2.30 |
| 480 | 1.7 | 5.14 | 530 | 2.30 |
| 490 | 1.2 | 3.48 | 550 | 1.95 |
| 500 | 2.3 | 0.53 | 585 | 1.40 |
| 510 | 3.4 | 4.70 | 620 | 1.30 |
| 520 | 7.2 | 7.10 | 630 | 1.00 |
| 530 | 7.3 | 2.00 | 650 | 1.00 |
| 540 | 6.7 | 0.60 | 700 | 1.00 |
| 550 | 5.2 | 0.50 | | |
| 560 | 4.1 | 0.70 | | |
| 570 | 1.7 | 0.60 | | |
| 580 | 1.1 | 0.90 | | |
| 590 | 1.1 | 2.10 | | |
| 600 | 1.6 | 3.50 | | |
| 610 | 2.5 | 2.10 | | |
| 620 | 5.0 | 0.60 | | |
| 630 | 6.0 | 1.00 | | |
| 640 | 11.5 | 9.50 | | |
| 650 | 14.5 | — | | |

they have been obtained from one and the same subject and under the same conditions as in the measurements of electrical excitability.

Two examples of hue discrimination data are reproduced in the second columns of Tables I and II, and the data in Table I are represented graphically in Fig. 2 ($\Delta\lambda$–curve). In this case (Motokawa), the minima of threshold wave-length difference $\Delta\lambda$ lie at about 460, 490, 585 and 630 mμ. Steindler found regions of minimal thresholds at 435, 495, 585 and 636 mμ for her own eye, and Laurens and Hamilton at 440, 490, 585 and 620 mμ for Hamilton's eye and at 440, 482, 578 and 630 mμ for Lauren's eye.

According to Steindler and others, these minima in hue discrimination curves can be explained on the basis of the sensation curves as studied by König and Dieterici, Exner and others. This is evidenced above all by the fact that the minima of the first order correspond to the intersection points of the sensation curves. It is true that some relation exists between sensation curves and hue discrimination curves, but the mechanism of hue discrimination will not be elucidated on the basis of this relation. The sensation curves are obtained from color mixing experiments in which color matching evidently is dependent on hue discrimination. In other words,

the sensation curves should be explained on the basis of hue discrimination, but the reverse is evidently irrational. It will be shown in the following that the hue discrimination finds its explanation in physiological processes in the retina.

## 2. Physiological Processes underlying Hue Discrimination.

Curves for the electrical excitability after illumination with spectral lights of various wave-lengths are shown in Fig. 1, in which excitability increases in percentage of the excitability in the dark are plotted as ordinates against intervals after the end of illumination as abscissae. Each curve of this figure was obtained in one sitting of about three to four hours. As stated in the preceding paper, the crest time, i.e. the time to the maximum is characteristic of the wave-length of light and remains constant so long as lights of any intensity but of one and the same wave-length are used for illumination. However, the height of the curve depends greatly upon the

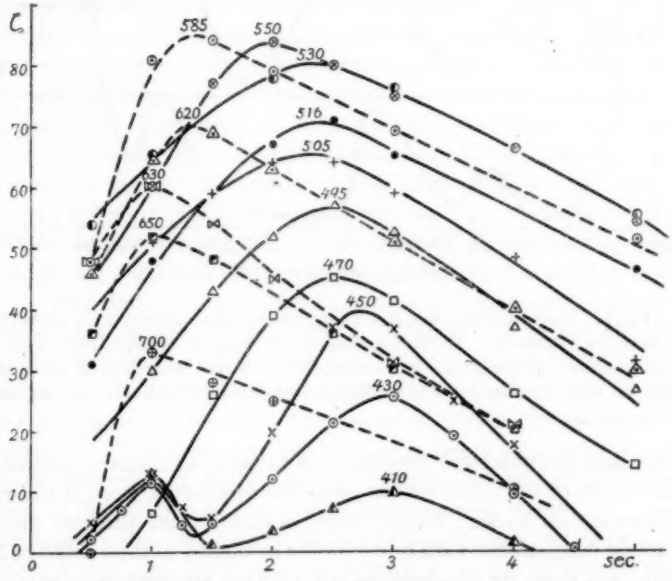


Fig. 1. Time course of electrical excitability after illumination with spectral lights of varying wave-lengths. Ordinates: Increases in electrical excitability due to illumination in percentage of the excitability without preceding illumination. Abscissæ: Time in seconds after the end of illumination. The number attached to each curve expresses wave-length in mμ. The crest time of each curve is shown in Table II.

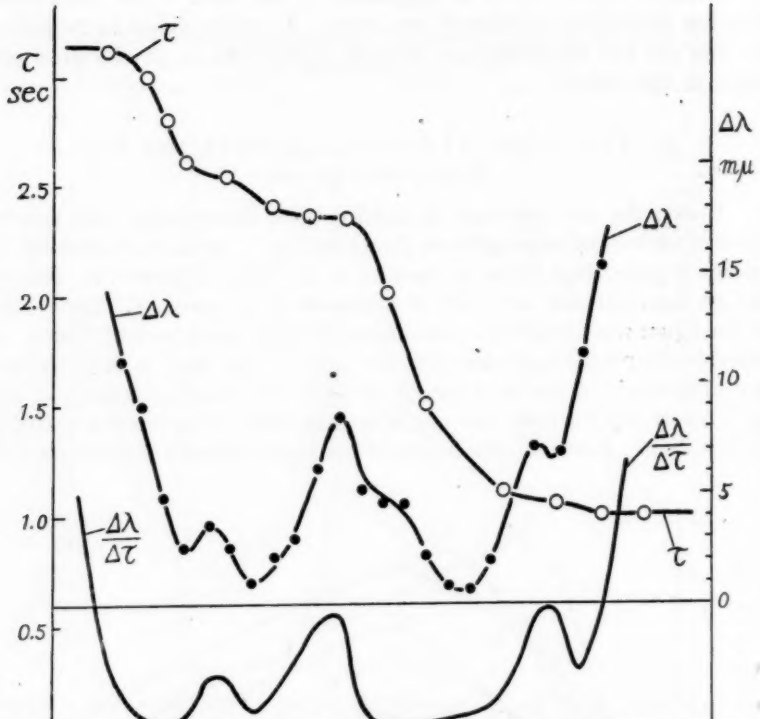


Fig. 2. Hue discrimination curve $\Delta\lambda$ and wave-length discrimination curve $\Delta\lambda/\Delta\tau$ as measured electrically. Curve $\tau$ represents the relation between crest time and wave-length $\lambda$. Curve $\Delta\lambda/\Delta\tau$ was constructed with the reciprocals of the slope of curve $\tau$. Data in Table I. The electrical curve $\Delta\lambda/\Delta\tau$ resembles the hue discrimination curve $\Delta\lambda$.

intensity and physiological conditions of the eye, so that we cannot always obtain curves of the same height in different sittings, even if the external conditions are identical. So the curves obtained from different sittings had to be made comparable with one another in regard to their relative heights, and this was done on the basis of the relation established in my previous work that two different curves for one and the same wave-length can always be brought into coincidence by some adequate vertical displacement of one of the curves as a whole. This sort of correction was made in the following way: The values of $\zeta$ corresponding to the respective crest times

were determined in one sitting, and the curves obtained in different sittings were vertically displaced until their maxima reached the values of $\zeta$ obtained in one and the same sitting. The curves illustrated in Fig. 1 are corrected in this way so that they are comparable with one another not only in their crest times but also in their heights.

It will be seen from this figure that the crest time increases with decreasing wave-length, while the height of the curves increases at first, reaches a maximum between 585 and 550 m$\mu$ and decreases with further shortning of wave-lengths. This change of the height is of great physiological importance, but will not be mentioned further, for it is little concerned with the present problem.

The values of crest times measured from these curves are shown in the last column of Table II. It is worth noticing that the curves for violet lights have two maxima. The crest time of one maximum coincides with that of the curve for red light, and the crest time of the other lies near that of the curve for blue light. This fact indicates that violet light is capable of exciting the same receptor system as that for red light, and is in good agreement with the psychological fact that violet is reddish blue.

For further corroboration of this relation another set of measurements was performed under similar experimental conditions. The data, however, are not represented graphically because the relation is quite similar to the above findings. The values of crest time obtained from these measurements are shown in Table I, and plotted as ordinates against wave-lengths as abscissae in Fig. 2. As seen from this figure, the curve of crest time $\tau$ has several inflexion points indicating that the rate of change of $\tau$ against $\lambda$ is never homogeneous over the entire range of the spectrum. The curve $\Delta\lambda/\Delta\tau$ in the same figure was drawn with the reciprocals of the slope of the curve $\tau$. The slope was measured by means of Yamamoto's differentiator, a convenient apparatus made at the Institute for Chemical and Physical Researches in Tokyo. It is surprising that the curve $\Delta\lambda/\Delta\tau$ represents a close copy of the curve for wave-length discrimination $\Delta\lambda$; it has four minima and three maxima about the same regions of the spectrum as does the curve $\Delta\lambda$. The same relation consists between both sorts of measurements reproduced in Table II, though a slight discrepancy exists as to the positions of the minima at the orange part of the spectrum.

This agreement cannot be a mere chance, because we have a number of evidence that the effect of wave-lengths is characterized by the time factor of peripheral excitation.

## Discussion.

In our experiments, we are evidently dealing with after-effects of illumination or recovery processes from the effect of illumination. So it

may seem at first sight that our phenomenon is concerned not so much with visual function during illumination as with after-images, but this is not the case. The most convincing evidence for this is that our electrical data agree very closely with hue discrimination data which were obtained clearly independent of after-images or temporal induction. After-images involve the central visual mechanism, which would be excited either by some after-effect remaining in the central nervous system itself or by centripetal impulses initiated by some after-effect in the periphery. After-effects which can be proved by our electrical method do not always initiate centripetal impulses, and consequently are not always related to any after-image. This is evidenced by the fact that a remarkable increase in electrical excitability can be proved after such a weak illumination as gives rise to no indication of after-image. Our phenomenon is an electrical expression of the after-effect in the periphery, and we study its amount and velocity of decay to get information of the changes during illumination.

Variations of crest time with varying wave-lengths indicate that some physiological mechanisms with varying time constants are excited by lights of varying wave-lengths. The question now arises whether there are as many kinds of processes as would correspond to the number of discernible hues in the spectrum, or whether there exist only a few mechanisms with different time constants, as is assumed in the theories of color vision. The fact that various values of crest time are obtainable seems to support the former view, but this fact can be explained by the assumption that our curves of electrical excitability represent summation effects of a few component processes. The experiments of summation reported in my preceding paper[1] serve for verification of this interpretation.

More conclusive are the findings on subjects of anomalous color vision. In these cases the summation of the component processes with different time constants is less complete probably because of unbalanced development of the processes, so that they hardly fuse together into a single congruent curve. The excitability curve has two or three maxima in such cases, and the crest times do not vary with variations of wave-lengths. Researches on this problem are now in progress in our laboratory, and further details will be reported in the near future.

## Summary.

The electrical excitability of the eye increases temporarily after illumination, and the time course of this variation is characteristic of the wave-length of the light used for illumination. The sensibility of the eye to wave-length differences was studied with the time constant of th eelectrical excitability as the index.

For a comparison, thresholds for hue discrimination were measured over the entire range of the spectrum. The curve for just perceptible differences in wave-lengths plotted against wave-lengths shows minima in the violet, blue-green, yellow and orange.

As the time constant of the electrical excitability after illumination was chosen the crest time which means the interval between the end of illumination and the maximum of excitability. The crest time increases in a characteristic manner as the wave-length $\lambda$ is shortened. If the decrease of wave-length $\Delta\lambda$ corresponding to a definite amount of increase in crest time $\Delta\tau$ is plotted against $\lambda$, a curve is obtained which represents a close copy of the curve for hue discrimination. This fact suggests that the physiological basis for hue discrimination lies in the periphery, as has been assumed in the theories of color vision.

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